Rapid and high yield Extraction method for Saponins from *Safed musli*

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ABSTRACT

Objectives: We aimed to develop, compare and optimise rapid and high yield extraction method for saponins of *Safed musli* using conventional extraction techniques and as well as modern microwave assisted solvent extraction method. **Materials and methods:** Roots of *Safed musli* (*Chlorophytum borivilianum*) are extracted by maceration, soxhlet, sonication and microwave methods. Extract further fractionated to obtain total saponins. Microwave assisted solvent extraction (MASE) method is optimised using Taguchi L9 orthogonal array design. Total saponins are estimated by High Performance Thin Layer chromatography (HPTLC) from all extracts obtained by different methods. **Results:** Factors namely temperature, irradiation time, irradiation power and powder size which potentially affects extraction efficiency are considered while optimizing MASE by statistical orthogonal array design procedure and saponins are quantified using HPTLC. Under developed optimum conditions, MASE showed significantly higher yield (5.11%) and drastic reduction in extraction time (4 min) than conventional extraction methods. **Conclusion:** Saponins of *Safed musli* shown highest yield in MASE and then maceration, soxhlet and sonication followed. The developed and optimised method of saponin extraction by MASE can have huge industrial applications after scale up.

Key words: HPTLC, Microwave assisted solvent extraction, Maceration Saponins, Orthogonal test L9 (34) Sonication, Taguchi Design.

INTRODUCTION

In Ayurveda, Siddha, Unani, *Safed musli* roots are very popular and well known for its aphrodisiac as well as immune-modulatory activity and hence it is important ingredient of 50 Ayurvedic and Unani preparations. *Safed musli* is also one of the important ingredients of very popular and useful Ayurvedic formula-Chyawanprash. Thirteen species of *Chlorophytum*, reported from India, sold as '*Safed musli*' in the Indian drug market. From research it is confirmed that the therapeutic effects of *Safed musli* are due to the presence of large amount of saponins. Among all species, *Chlorophytum borivilianum* produces the highest yield and highest saponin content. Its International drug market value is more than 300-700 tons per year. But factors like poor seed germination and dormancy are affecting uniform supply of this musli in market.[1] A solution to overcome such situation is the development of Rapid and high yield extraction method in order to obtain valuable metabolites. Traditionally the very common method for extraction of this saponin has been Soxhlet extraction. But the Soxhlet extraction method requires long heating time, bulk amount of organic solvents which again involves high risk of thermal decomposition of drug substances and pollution.[2] Despite of large preference to this method, researchers needs new fast and reliable methods of extraction. Microwave assisted Solvent extraction[3] offers simultaneous heating of sample material and solvent to obtain improved yield. The principle of MASE depends on dielectric properties of the solvent as well as of matrix where cell bursting is caused due to localized internal superheating followed by penetration of solvent into matrix and thus dissolution of the active components.[2] This surely enables improved and selective extraction of active phytochemicals with less time.[4] Hence the present work is reporting a new MASE method for fast and efficient extraction of Saponins from the roots of *Chlorophytum borivilianum* and comparison with conventional extraction techniques and optimization using Taguchi L9 orthogonal array design.[5]

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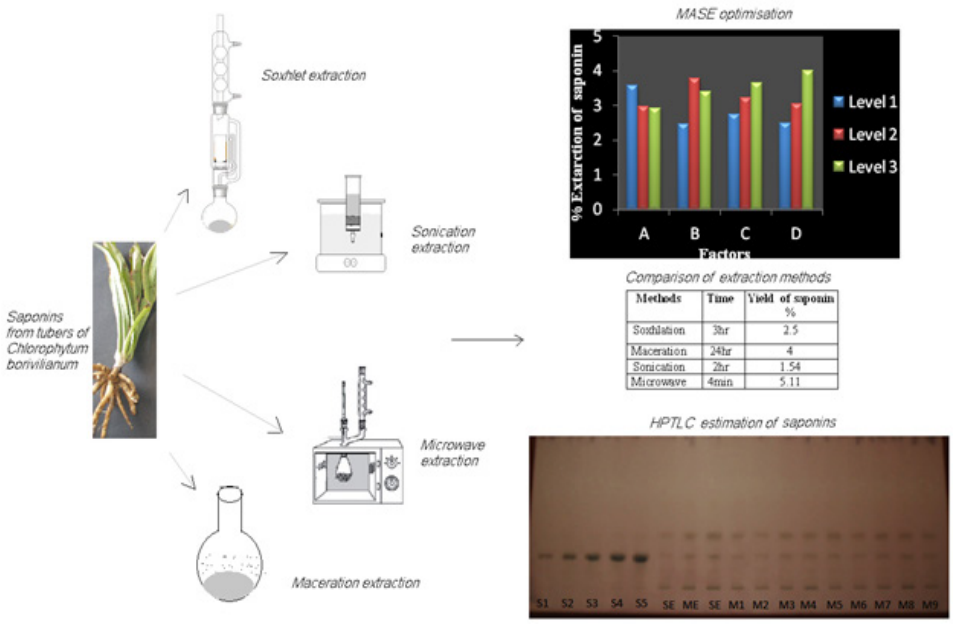

Graphical Abstract

MATERIALS AND METHODS

Plant materials

Chlorophytum borivilianum roots were purchased from local cultivator and authenticated by Head, Botany Department, Government Vidarbha Institute of Science and Humanities, Amravati). The roots were dried, powdered and defatted by petroleum ether and sieved through mesh size #22, 44, 60.

Reagents and Apparatus

All solvents used were of analytical grade. Percolated silica gel 60F254 plates for HPTLC analysis were obtained from E. Merck. Standard Saponin was isolated from tubers and confirmed in previous publication.[6] The microwave extractor was from Catalyst Systems (Pune, India). A HPTLC system was from Camag. A normal 500 ml capacity glass Soxhlet extractor was used Soxhlet extraction. Maceration was performed in 500 ml capacity glass maceration apparatus. Sonication extractor was from Spectralab Pvt. Ltd.

Extraction of Saponins

Soxhlet extraction

Soxhlet extraction was performed using a classical glass Soxhlet apparatus of capacity 500 ml. The tubers were washed, dried, powered and defatted with petroleum ether. 15 g powder (screened through mesh 22) was extracted for 3 hr with 300 ml. Finally extract was evaporated to dryness under vacuum.

Maceration extraction

Cold maceration extraction of 3 g sample (# 22) with 60 ml methanol was carried out in a closed glass conical flask for 24 hr. The suspension was filtered and filtered extract were evaporated to dryness under vacuum and processed further.

Sonication extraction

Sonication extraction was carried out in sonicator for 2hr. In this method, sound waves of high frequency pulses of 20 kHz are generated in an ultrasonic bath. The waves generated from transmitter easily penetrate the cell membrane by inducing a mechanical stress on the cell. This increases cell wall permeability causes solubilisation of maximum amount of active constituents.[7] Heat was not applied in this method. 3 g powdered drug sample (screened through sieve 22) was extracted with 60 ml methanol. The suspension was filtered and filtered extract were evaporated to dryness under vacuum and processed further.

Microwave Assisted Extraction

3 g of powder was placed in MASE vessel along with 60 ml of methanol solvent. Then vessel placed inside the microwave cavity and MASE was carried out at different power levels for different irradiation time, irradiation temperature, and different particle sizes. After extraction,

Table 1: Results of the orthogonal design (A-D are code)

Level	Microwave power (%) A	Irradiation temperature B	Irradiation time, (min) C	Sieve Number D
1	20	60	1	22
2	60	50	2	44
3	100	40	4	60

Table 2: Results of orthogonal test L9 (3^4)

Tests	A	B	C	D	Saponin (mg)	% Saponin
1	1	1	1	1	50.1	1.67
2	1	2	2	2	120.1	4.00
3	1	3	3	3	153.3	5.11
4	2	1	2	3	94	3.13
5	2	2	3	1	98.1	3.27
6	2	3	1	2	76	2.53
7	3	1	3	2	76.5	2.54
8	3	2	1	3	112.5	3.75
9	3	3	2	1	75.2	2.50

extracts were filtered and evaporated under vacuum to dryness.

Optimization method

MASE of Saponins from *Chlorophytum borivilianum* has been optimized using Taguchi-based optimization technique.[8] In this method minimum number of experiments required for accurate optimization reduces time and cost of extraction. All the results are mean of triplicate experiments. In the present work, three levels i.e. K1, K2, K3 are allotted for each of the factors as shown in Table 1. A L9 orthogonal array scheme which requires 9 experiments for optimization process was performed and extraction results are summarized in Table 2. The optimum level for each factor was determined from the graphical representation of the analysis of mean values from each level for a particular factor.

RESULTS AND DISCUSSION

Extraction

Extraction dried root powder was extracted by different conventional extraction techniques (soxhlet, sonication and maceration) and MASE as per our previously reported method where Methanolic extract fractionated by diethyl ether to give solid precipitate of saponins. Further extraction efficiency was reported by comparing yield of all methods.

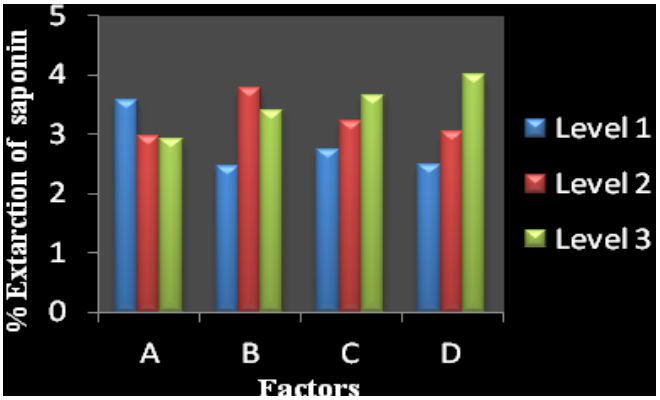

Figure 1: Graph showing Percentage extraction of saponin, obtained under orthogonal condition of MASE

Percentage extraction of Saponin = mass of saponin in crude extract ×100/ mass of raw material. A=Microwave power [level1=20%; level2=60%; level3=100%]; B=Irradiation temperature [level1=60°C; level2=50°C; level3=40°C] C=Irradiation time [level1=1min; level2=2min; level3=4min]; D=Sieve number [level1=22; level2=44; level3=60].

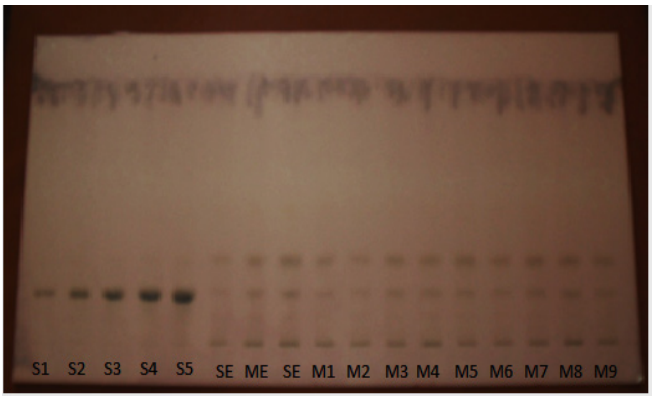

Figure 2: HPTLC chromatogram obtained by different extraction methods

S1-S5 = Standard compound spots; SE= Soxhlet extraction; ME= Maceration extraction; SE=Sonication extraction; M1= MASE Test1; M2= MASE Test2; M3= MASE Test3; M4= MASE Test4; M5= MASE Test5; M6= MASE Test6; M7= MASE Test7; M8= MASE Test8; M9= MASE Test9.

Optimization of microwave extraction conditions

As microwave extraction has been carried out for the first time it requires optimization for its different parameters which was done using an orthogonal array design. The factors were microwave power (A), irradiation temperature (B), irradiation time (C) and particle size (D). For each variable, the influence on the yield of saponin was considered from three levels. Figure 1 has been constructed based on the mean values obtained for each level from a particular factor to know influence of each variable on the extraction result. Figure 1 show when microwave power level was increased from 20% to 100% there was 0.66% decrease in amount of saponin. It has been also observed that the highest yield of saponin (5.11%) obtained when the sample was extracted with methanol at 50°C and yield was decreased at 60°C. Graphical representation of the analysis of means Figure 2 indicates that 20% microwave power

Table 3: Average yield of Saponin at different levels for each factors

Levels	A	B	C	D
K1	3.59	2.44	2.65	2.48
K2	2.97	3.67	3.21	3.02
K3	2.93	3.38	3.64	3.99

Table 4: Comparison of MASE with conventional extraction methods

Extraction methods	Extraction time	Solvent consumption (ml/g)	Yield of saponin (%)
Soxhlet	3 hr	20	2.5±0.1%
Maceration	24 hr	20	4±0.2 %
Sonication	2 hr	20	1.54±0.1%
MASE	4 min	20	5.11±0.3%

and irradiation temperature of 50°C were ideal to obtain maximum saponin content. Because it has been observed that with high microwave power and high irradiation temperature there might have been intense internal superheating of the plant matrix resulting in degradation of the active constituents which because of polar nature is more prone to damage due to intense microwave heating.[8]

Maximum amount of saponin was obtained with irradiation time of 4 min and yield of was dropped saponin slightly when irradiation time was increased to 2 min (Figure 2) This indicates that a irradiation time of 4 min is sufficient to bring about the extraction of saponin. Powder mesh size # 60 gave higher amount of saponin followed by # 44 and #22 Figure 2. This indicates that powder of fine mesh size 60 is suitable for extraction. With the use of fine particles, microwaves will be facilitated with deep penetration ability resulting in thermal degradation of active constituents.

From Table 3, the optimized experimental conditions are: microwave power- 20%, irradiation temperature - 50˚C, irradiation time- 4 min and powder of mesh size-60. It is possible to enhance the yield of Saponin using a different levels combination of factors found at optimum conditions.

Comparison of MASE and other conventional techniques

The selection of extraction methods mainly depends on the advantages and disadvantages of the processes such as extraction yield, complexity, production costs, environmental friendliness and safety.[9,10] In general soxhlet, maceration and sonication are the most commonly used extraction methods. The drawbacks of soxhlet, maceration and sonication are the large amount of solvent and long extraction time needed.[11] Compared with conventional extraction methods MASE method is selective, rapid, efficient, and economic. The results of comparison of MASE with conventional extraction methods for extraction of saponins from *Chlorophytum borivilianum* roots are shown in Table 4 in terms of amount of saponin. Highest yield of Saponins in less time was obtained by MASE which confirms reliability of this method.

Saponin Quantification by HPTLC

In HPTLC analysis, samples were spotted (2 µl) in the form of bands of width 6 mm, positioned 10 mm from the bottom of the plate, with a Camag microlitre syringe (100 ul) on precoated Silica gel F^{254} (20 cm×10 cm). The mobile phase used is chloroform: glacial acetic acid: methanol: water (16:8:3:2, v/v/ v). Linear ascending development for 30 min at room temperature in a twin trough glass chamber up to the height of the solvent front 80 mm was carried out. Quantification was done by Camag TLC scanner III at 264 nm. Standard solution (10 µg/µl in methanol) volumes of 1–5 µl were used corresponding

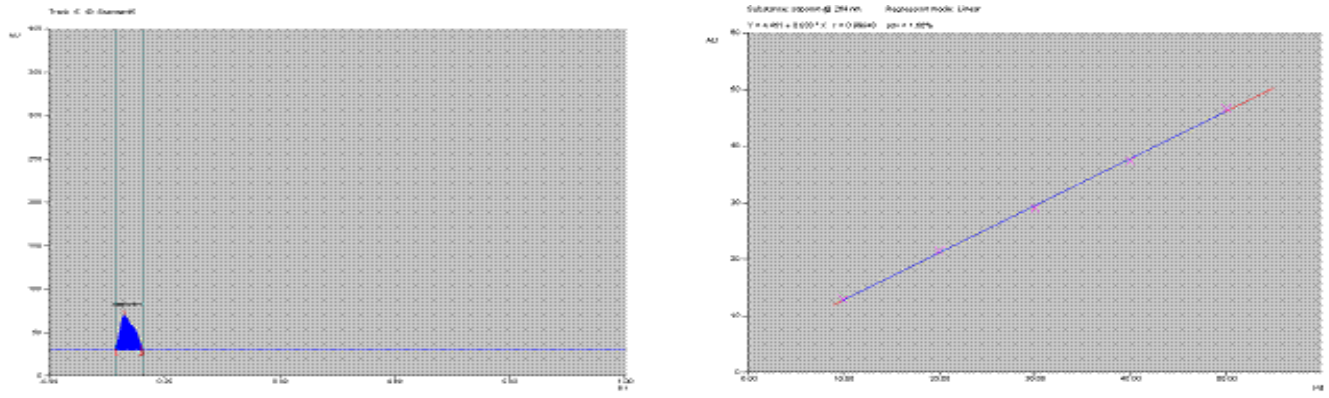

Figure 3: Chromatogram of standard Saponin and Calibration curve for total saponin content determination

to an amount of 1000–5000 ng. The different extracts obtained from extraction methods were subjected to HPTLC fingerprinting studies where, the concentration of standard solution was (10 µg/µl in methanol). The fingerprinting pattern of different extracts of *Chlorophtum borivilianum* roots are shown in Figure. 2 and 3. All extract are compared qualitatively as well as quantitatively by HPTLC fingerprinting in respect to the number and quantity of phytoconstituents present in each extracts, It has been found that maximum amount of saponins content is present in extract obtained by microwave assisted extraction. Track 9 and track 10 shows maximum area and it has shown higher quantity of saponin. Other parameters like percent yield, extraction time and solvent quantity are also compared. It has been found that, microwave assisted extraction method is effective in terms of time, quality and quantity of the saponins.

CONCLUSION

From this study it is explored that microwave extraction is most suitable extraction method for fast and exhaustive isolation of maximum amount of medicinally important saponins from tubers of *Chlorophtum borivilianum* as compared to maceration, soxhlet and ultrasound methods. Optimization method of orthogonal array design in case of MASE shows that the amount of saponins extracted is highly dependent on microwave power, irradiation temperature, irradiation time and matrix characteristics. Comparative extraction time and percentage of saponin fraction also confirms that MASE is the fastest and efficient extraction method among all extraction methods. Future scope involves industrial scale-up and necessary commercialisation of this novel method.

CONFLICTS OF INTEREST

Authors do not have any conflict of interest.

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Highlights of Paper

- *Safed musli* roots are very popular and important ingredient of more than 50 Ayurvedic and Unani preparations.
- Saponins are active constituents responsible for therapeutic efficacy of roots.
- Most of species of *Safed musli* are under endangered or rare list of biodiversity survey.
- Developed microwave assisted extraction method is rapid and allows exhaustive isolation of total saponins.

Author Profile

- **Dr. S. L Deore** is GOLD MEDALIST in B.PHARM from North Maharashtra University in 2004. She has completed M.Pharm from Govt. College of pharmacy, Amravati in 2006. She has awarded PhD in pharmaceutical sciences by SGB Amravati University in May 2011. She has isolated two saponins in her PhD work. She has published 35 research papers in various national- international journals about the medicinal plants and herbal drugs. She has authored two books "Plant biosynthesis" and "Experimental Phytopharmacognsoy" for Nirali Prakashan, Pune. Recently she has published Textbook of Pharmacognosy by PharmMed Press, Hyderabad. Her areas of research are isolation and structural elucidation of phytochemicals, neutraceutical development, and traditional medicine screening, chromatographic and phytochemical analysis of extracts.

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Taste Masking of Ayurvedic Nutraceutical Formulation by Pan Coating Process

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ABSTRACT

Background: Ayurvedic medicines and nutraceuticals are gaining popularity among physicians and patients for better therapeutic value. Lack of quality standards and problems, in preparing or testing them, are the main hurdles experienced by both the practitioners and the patients. **Objective:** The objective of the study was to improve the palatability of the Ayurvedic Nutraceutical Preparation (ADS) by masking its bitter taste and to standardize the taste masking procedure. In the present study Eudragit E 100 was used as an acid soluble coating material. **Materials and Methods:** ADS powder was converted into granules with PVP K30 as a granulating agent and the ADS granules were coated with Eudragit E 100 coating solution by pan coating process. Various IPQC tests namely flow properties, moisture content were performed on the granules before and after coating for determination of endpoint of granulation and coating respectively. The ADS powder and granules were evaluated for bitter taste. **Results:** ADS granules were advantageous over ADS powder since the flow properties of ADS granules were better than the flow properties of ADS powder, a prerequisite of pan coating process. Eudragit E 100 inhibited the contact in between the plant extracts and the taste buds due to insolubility of Eudragit E 100 in saliva. Sensory evaluation of taste indicated that the taste of coated granules was significantly masked. **Conclusion:** The bitter taste of ADS was improved successfully with Eudragit E 100 as a coating agent and the pan coating process. An attempt was made to standardize the pan coating process.

Key words: Ayurveda, Coating, Eudragit E 100, Nutraceutical, Taste Masking.

INTRODUCTION

Ayurvedic literature suggests use of herbal, mineral and herbo-mineral preparations in various dosage forms for centuries. Traditional knowledge inspired Dietary Supplements are nowadays being promoted for their perceived health benefits. The major challenge for the ayurvedic nutraceutical industry in using the medicinal plant extracts is the palataibilty of extracts.[1]

Plants protect themselves against herbivorous animals by producing secondary metabolites namely, phenols, flavonoids, isoflavones, terpenes, and glucosinolates. The secondary metabolites, having bactericidal or biological activity, are used therapeutically. These substances are almost always bitter, acrid, or astringent and may provide a defense against predators by making the plant unpalatable.[2]

Taste masking is defined as a perceived reduction of an undesirable taste that would otherwise exist.[3] Molecule interacts with taste receptors, in taste buds, on the tongue to give bitter, sweet or other taste sensation, when it dissolves in saliva. The taste buds contain very sensitive nerve endings, which produce and transmit electrical impulses via the seventh, ninth and tenth cranial nerves to those areas of the brain, which are devoted to the perception of taste.[4]

The taste masking includes physical and chemical means that prevent the interaction of the drug substance with the

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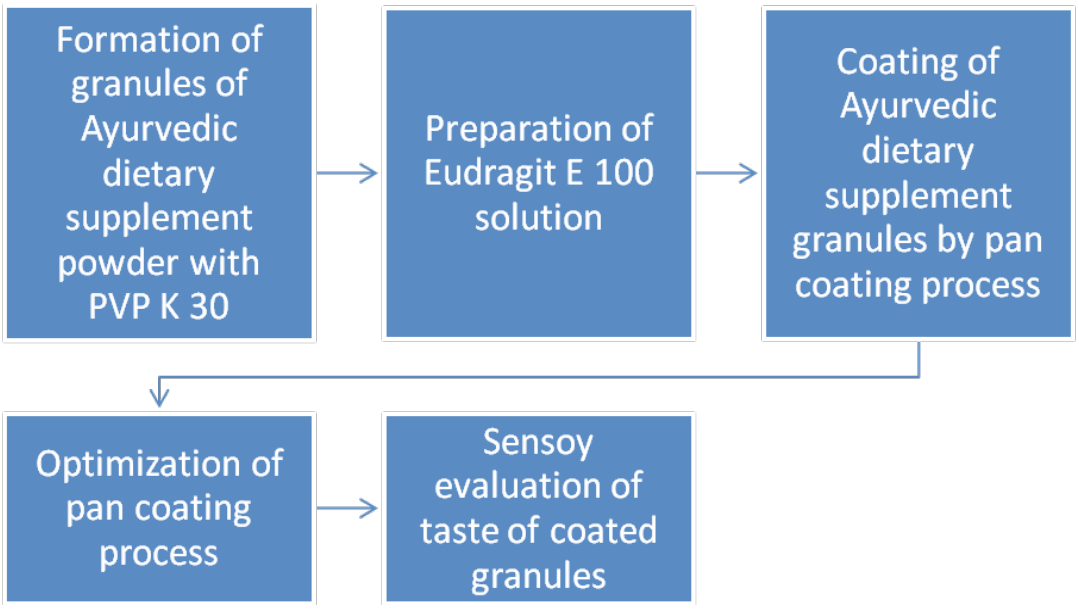

Graphical Abstract

taste buds. The commonly employed methods for achieving taste masking are use of flavor enhancers, coating of drug particles with inert agents, taste masking by formation of inclusion complexes and molecular complexes, microencapsulation of drug, formation of solid dispersions, multiple emulsions of drug, use of ion exchange resins and prodrugs, drug entrapment in liposomes, taste masking by viscosity modifications.[5]

An ideal taste masking process and formulation should involve least number of equipments and processing steps. It should require minimum number of excipients for an optimum formulation. The taste masking process should not affect the bioavailability of the drug adversely. The excipients, used for taste masking, should be economical, easily available and should have high margin of safety.[4]

Eudragit E 100 (Figure 1) is a cationic copolymer of (2-dimetylaminoethyl) methacrylate, butyl methacrylate, and methyl methacrylate in the ratio of 2:1:1. It is used

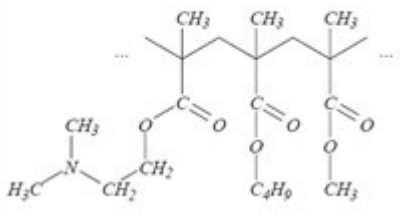

Figure 1: Eudragit E 100

frequently for taste masking since it is soluble up to pH 5; however it is swellable and permeable above pH 5.8. Eudragit E 100 is insoluble in saliva; however it becomes water soluble at gastric pH via salt formation with acids thus providing gastro soluble coatings. Eudragit E 100 coatings are moisture protective due to very low water vapor permeability. Moreover, good storage stability, protection of sensitive actives and improved passage of the dosage form across gastrointestinal tract (GIT) are further advantages of Eudragit E 100 coatings. No plasticizer is needed for Eudragit E 100 as it is soft enough to build flexible coatings.[6,7]

An ayurvedic dietary supplement (ADS), consisting of medicinal plant extracts,was reported as bitter in taste by diabetic patients.The objective of this investigation was to improve the palatability of the preparation for better compliance from the end user. The study was focused at masking the bitter taste of ADS by preventing the contact of ADS with taste buds, without affecting its absorption from the GIT. Eudragit E 100 was applied as a coating agent by pan coating process. Another important objective was standardization of the pan coating process.

MATERIALS AND METHODS

Ayurvedic dietary supplement in powder form was procured as a gift sample from Vaidya Ajit Kolatkar Pune, India. Eudragit E 100 and polyvinyl pyrrolidone K 30 (PVP K 30) were purchased from Anil Suppliers, Pune, India. All other ingredients used were of analytical grade.

Table 1: Coating parameters for coating of ADS granules

Coating Parameter	Value
Pan speed	16-20
Pump rpm	1
Inlet temperature	50-55ºC
Atomization air pressure	Low
Spray rate	3-5 ml/min

Characterization of ADS powder

The ayurvedic dietary supplement (ADS) powder was evaluated for the physical properties namely, color, appearance, taste, particle size, bulk density, tapped density (Electrolab Tap density tester USP ETD-1020), compressibility index, Hausner's ratio and the angle of repose.[8] Bulk density was determined by the IP method I; tapped density was determined by tapping the sample 500 times. The moisture content of ADS powder was determined.[9]

Preparation of ADS Granules

PVP K30 was used as a granulating agent for converting ADS powder to ADS granules. ADS powder (50 gm) was mixed with 5% w/v aqueous solution of PVP K30 (30 ml) in a glass pestle mortar for 5 minute manually to form a cohesive mass. The cohesive mass was sieved through sieve 10 to obtain the granules. The granules were dried at 60°C for 2 hours.

In Process Quality Control (IPQC) Tests for ADS Granules

The ADS granules were evaluated for the physical properties namely, bulk density, tapped density, compressibility index, Hausner's ratio and the angle of repose.[8] The moisture content of ADS granules was determined.

Preparation of Coating Solution

The coating solution of Eudragit E 100 (70 ml) was prepared by dissolving Eudragit E 100 (0.025% w/v) in ethanol (99% v/v) with gentle stirring, on a mechanical stirrer at 50 rpm for 10 minute, till a clear solution was observed. The coating solution was passed through 100 mesh screen and used for coating.[10] The relative density of coating solution was determined.[9]

Coating of ADS granules

The ADS granules were coated by pan coating process with the coating parameters.[11] (Table 1) A side-vented Accela Cotta R & D Tablet coating machine, (Ideal Cures, Mumbai) having a pan diameter of 4"and fitted with a spray gun, was used to coat the ADS granules. ADS granules (50 g) were introduced into the coating pan of the Accela tablet coater rotating at a rate of 10-16 revolutions per min (rpm). The exhaust for dry air was turned on and the temperature of the set up was allowed to reach the maximum of 55°C. Before application of coating solution, the granules were pre-warmed at 55ºC for 10 minutes at slow rpm or by inching. The nozzle of the spray gun was directed at the centre of the rotating pan, the gun height was adjusted at 25 cm and Eudragit E 100 coating solution was pumped continuously on to the rotating granule bed at a spray rate of 3-5 ml/min. The coating was dried by allowing hot air intermittently on the rotating granule bed (drying period 5 min). The ADS granules were stirred during drying to prevent aggregation. After coating, the granules were post dried for 10 to 15 minutes at slow rpm or by inching. The coating solution was applied to the granule bed till the weight gain was 3% w/w. For 50 g of ADS granules, 58 ml of coating solution was added. The duration of coating was 3 hours.

In Process Quality Control (IPQC) Tests for Coated ADS Granules

The ADS coated granules were evaluated for angle of repose, bulk density, tapped density, compressibility index, Hausner's ratio to determine the flow properties.[8] The moisture content and the residual solvent (alcohol) in the coated granules was determined.[9]

Evaluation for Taste Masking

Sensory evaluation of bitter taste of ADS powder, uncoated and coated ADS granules was determined by a single blind cross-over study based on taste recognition by six volunteers from whom informed consent was obtained.[12] The volunteers were instructed to keep the ADS powder, uncoated and coated ADS granules in the centre of the tongue and not to swallow the powder or the granules. The powder/ granules were retained in the mouth for 30 seconds, and then the mouth was thoroughly rinsed with distilled water. The response of the volunteers was recorded on the bitterness scale (0=good, 1=tasteless, 2=slightly bitter, 3=bitter, 4=very bitter).

RESULTS AND DISCUSSION

Characterization of ADS powder

The ADS powder was yellowish and dry. The particle size was 250 μ since the powder was passed through sieve 60.

Table 2: Flow properties of ADS granules

Property	ADS powder	ADS Uncoated granules	ADS Coated granules
Angle of repose	49.28°	43.28°	36.51°
Bulk density	0.204 g/ml	0.377 g/ml	0.491 g/ml
Tapped density	0.328 g/ml	0.512 g/ml	0.585 g/ml
Carr's index	37.8%	26.25%	16.06%
Hausner's ratio	1.60	1.35	1.19
Moisture content	13.62%	34.41%	9.38%

According to angle of repose-compressibility-flowability correlation data, if angle of repose is 46-55°, it means poor flow property. If compressibility index and Hausner's ratio is 31-37 and 1.46-1.59 respectively, it means very poor flowability. The ADS powder revealed very poor flow properties.[8] (Table 2)

Preparation of ADS Granules

Taste masking of ADSpowder was performed in 2 stages, namely preparation of granules of ADS powder and coating of ADS granules with a polymer solution.[13] Powders, especially herbal in origin, are bitter in taste. Since powders have smaller particle size, coating of individual particle and masking of bitter taste is expensive and time consuming. Granules are advantageous over powders since they have better flow properties, compressibility, physical and chemical stability, particle size uniformity as compared to powders. Granules have a smaller surface area than a comparable volume of powder leading to better wettability. Granules require less coating solution and can be coated uniformly.[14] ADS powder was converted into granules.

PVP is a widely used excipient for the preparation of solid dosage forms. It is readily soluble in water and other polar solvents. PVP K 30 is a very good binder for effervescent tablets, as it dissolves rapidly in water to form a clear solution. Wet granulation with PVP K-30 generally gives hard and compact granules with good flow properties. Due to high binding strength, PVP K 30 is suitable for wet granulation and direct compression. PVP K 30 was used as a granulating agent for preparation of ADS granules.[15,16]

In Process Quality Control (IPQC) Test for ADS Granules

IPQC tests are checks performed during production in order to monitor and, if necessary, to adjust the process, to ensure that the product conforms to its specifications. In-process controls are usually performed within the production area and are accomplished by identifying the critical steps in manufacturing and controlling them within defined limits. In-process inspection and testing is performed by monitoring the process or by actual sample analysis at defined locations and times. In general, the in process control procedures are usually rapid and simple tests or inspections that are performed, when the manufacturing of a product batch is in progress. IPQC tests ensure the uniformity, purity and quality of finished dosage forms within a batch and between batches.[17]

The flow properties and moisture content of ADS granules were studied for determination of endpoint of granulation, in turn for standardization of granulation. (Table 2) Adequate flow properties ensure uniform coating of the granules by the coating agent in subsequent stage. According to angle of repose-compressibility-flowability correlation data, if angle of repose is 41-45°, it means passable flow property. If compressibility index and Hausner's ratio is 26-31 and 1.35-1.45 respectively, it means poor flowability.[8] ADS granules exhibited poor flow properties. If the moisture content of granules is too high, the granules are wet resulting in improper coating. If the moisture content is too low, the granules are friable and the particle size will reduce in the coating pan. In the present study, ADS granules exhibiting poor flow properties and moisture content of 34.41% were used for further study.

Coating of ADS granules

Eudragit E 100 (Aminoalkyl methacrylate copolymer) dissolves under acidic conditions/low pH but not at neutral pH. It becomes water-soluble via salt formation with acids, thus providing gastrosoluble film coatings. These films are soluble below pH 5 and swellable and permeable above pH 5. The pH of saliva ranges from 6.2 to 7.4. Ishikawa *et al* concluded that the taste masked granules can be prepared with Eudragit E 100.[10] In the present study Eudragit E 100 was used as an acid soluble coating material. The coating of Eudragit E 100 on ADS granules, prevented the contact in between the plant extracts (dry form) and the taste buds due to insolubility of Eudragit E 100 in saliva. Although dissolution of ADS granules in stomach was not examined *in vitro* or *in vivo*, it appears that rapid dissolution would occur in GIT.

The relative density of alcoholic solution of Eudragit EPO was 0.811. Drennew *et al*, concluded that in-process weighing of known sample size/weight gain is the evaluation test that determines the coating process endpoint.[18] The weight gain for ADS coated granules was 4.76% w/w.

In Process Quality Control (IPQC) Test on ADS coated granules

The flow properties of ADS coated granules were studied for determination of endpoint of coating, in turn for standardization of coating. (Table 2) Adequate flow properties ensure uniform filling in the final container. According to angle of repose- compressibility- flowability correlation data, if angle of repose is 36-40°, it means passable flow property. If compressibility index and Hausner's ratio is 16-20 and 1.19-1.25 respectively, it means fair flow properties.[8] The flow properties of ADS coated granules were improved over uncoated ADS granules. The ADS coated granules complied with the limit for residual solvent.[9]

Evaluation for Taste Masking

The taste masking study in human volunteers of ADS powder, the uncoated and coated ADS granules revealed significant masking of the bitter taste of ADS powder.[19] The taste of ADS powder was reported as 'very bitter' by 5 volunteers and as 'bitter' by 1 volunteer on the perception scale. All the 6 volunteers reported the ADS coated granules as being 'good' on the perception scale whereas 4 volunteers reported ADS uncoated granules as 'bitter' and 2 volunteers reported as being 'very bitter' on the perception scale. Moreover all the volunteers experienced a good mouth feel of the ADS coated granules. Thus sensory evaluation indicated that coating with Eudragit E 100 significantly improved the palatability of ADS formulation.

CONCLUSION

In conclusion, we succeeded in masking the bitter taste and in improving the palatability of medicinal plant extract based dietary supplement. An attempt was made to standardize the pan coating method for a suitable dosage form, coated granules, consisting of herbal powders. The present work suggested that the use of sweetners or other additives was not necessary for taste enhancement of ayurvedic nutraceutical formulation, especially for diabetic patients. The study would bring a paradigm shift in better compliance in users as well as better acceptance of ayurvedic dietary supplements by the nutraceutical industry. The preparation method designed in the present work is useful for masking the bitter taste of ayurvedic formulations.

CONFLICTS OF INTERESTS

The contributing authors do not have any conflicts of interest.

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Highlights of Paper

- The bitter taste of Ayurvedic dietary supplement was improved successfully with Eudragit E 100 as a coating agent and the pan coating process.
- Eudragit E 100 inhibited the contact in between the plant extracts and the taste buds due to insolubility of Eudragit E 100 in saliva.
- Sensory evaluation of taste indicated that the taste of coated granules was significantly masked.
- An attempt was made to standardize the pan coating process.

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Elucidation of β-sitosterol from *Benincasa hispida* Seeds, *Carissa congesta* Roots and *Polyalthia longifolia* Leaves by High Performance Thin Layer Chromatography

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ABSTRACT

Background: Fruits of *Benincasa hispida* (BH) is regarded as Valliphala due to its vast plethora of medicinal properties, *Carissa congesta* (CC) is an imperative local plant particularly in rural communities and *Polyalthia longifolia* (PL) is an ornamentally significant traditionally relevance plant in India system. β-sitosterol, an active constituent identified from enormous plants has been reported to possess excellent amount of pharmacotherapeutic potential by number of researchers. **Objective:** In the recent studies, the research team focuses on determining the percentage of the β-sitosterol present in the BH seeds, CC roots petroleum ether extracts as well as PL leaves ethanolic extract by chromatographic technique in harmony with High Performance Thin Layer Chromatography. **Materials and Methods:** Respective parts of BH, CC and PL plants were shade-dried and extracted by appropriate extraction methods followed by identification of β-sitosterol from the extracts by High Performance Thin Layer Chromatography after preliminary phytochemical screening extracts for the constituents. **Results:** The amount of β-sitosterol present in the BH seeds, CC roots and PL leaves extracts was found to be 23.00, 5.94 and 1.81 % w/w respectively. Research studies elucidated a peak that coincided with standard peak of β-sitosterol suggesting the presence of constituent in the extracts. **Conclusion:** Thus, extracts contains important constituent of β-sitosterol in BH, CC and PL.

Key words: *Benincasa hispida*, *Carissa congesta*, HPTLC, *Polyalthia longifolia*, β-sitosterol.

INTRODUCTION

Plant kingdom is regarded as an enormous reservoir of biologically active molecules but least fraction of plants with medicinal importance and activity are explored until date. Phytochemistry is one of the burgeoning fields of research, which has providing immense tool development in correlating data of the plant extracts. The progress has aided immensely by the development of rapid and accurate methods of screening plants for particular constituents present in the extracts.[1] Isolated and characterized phytoconstituents present in the plants have paved the way in contributing to healthcare and income to the consumer as well as government in terms of well- being and cheap price respectively.[2]

Benincasa hispida (Thunb.) Cogn. has been reported as Kushmanda Avaleha, Vasakhanda, Khanda and Rasayana in classical medicine in India.[3,4] Rasayana properties of this plant species is attributed in offering immune protection and is advised during the later years of life at forty-five in

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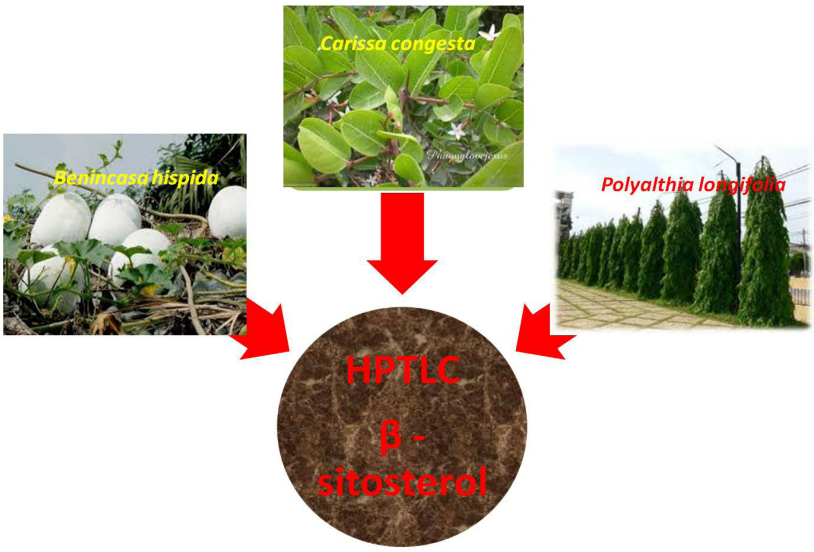


Graphical Abstract

Uses: *Benincasa hispida*, *Carissa congesta* (CC) and *Polyalthia longifolia* are significantly important plant in India system. These plants are well known among trial communities. The plants were shade-dried and extracted by appropriate extraction methods followed by identification of β-sitosterol from the extracts by High Performance Thin Layer Chromatography.

both the sexes.[5] The fruits has been found to contain ample quantity of proteins, enzymes, vitamin B_1 and C, flavonoid C-glycoside, terpenes, phenolic acids and free sugars as glucose, rhamnose, mannitol, uronic acid, trace metals, peptic polysaccharides (sequential extraction)[6,7] whereas phenolic compounds as astilbin, catechin and naringenin (high-speed counter current chromatography).[8-10]

Carissa congesta is well known in local people in India among the tribal communities due to its multiple properties as well as whole plant is powdered, mixed with cow's milk and given in diabetes.[11,12] The plant has been known to produce pentacyclic triterpenoids and glycosides yielding oderside H, digitoxigenin and sugars like D-glucose and D-digitalose.[13] The roots have yielded a various volatile principles like 2-acetylphenol, lignans as carinol, and a mixture of sesquiterpenes as carissone and carindone. Des-n-methylnoracronycine has been identified with constituents such as lupeol.[14-16]

Polyalthia longifolia is has common flowering plant in India with leaves used mostly in traditional medicine as an aromatic and essential oils due to its usefulness in various disorders.[17-19] Numbers of chemical constituents has been identified from the leaves such as azafluorene alkaloid and three new Aporphine N-oxide alkaloids.[20] Activity guided fractionation has elucidated presence of quercetin, quercetin-3-o-β-glucopyranoside, kaemperfol-3-o-α-rhamnopyranosyl-β-glucopyranoside,kaempferol-3-o-α-rhamnopyranosyl-(1-6)-β-gluco-pyranoside, rutin, β-sitosterol and allantoin from butanol fraction. Ethanolic extract has been found to contain bulbocapnin and steroids like β-sitosterol, stigmasterol and campesterol constituents.[21]

However, literature reports several methods for scrutinaztion of β-sitosterol from various plants species, no studies on HPTLC guided fraction for β-sitosterol from these plant extracts has been noticed. Selection of BH, CC and PL species for present research studies was based on its easily availability in local market and enormous pharmacological potential. Minute doses of β-sitosterol have been reported to increase the in vitro proliferative activity of T-lymphocytes as well as acting as chemopreventive agent in colon and breast cancer.[22,23] Taking pictograph of above viewpoints, our research article focuses on β-sitosterol percentage estimation in the BH seeds, CC roots petroleum ether, and further on PL leaves ethanolic extracts.

EXPERIMENTAL

Part A: Collection, authentication and extraction

All the studies undertaken in Part A have been previous reported by us in identification of lupeol from these extracts.[24-26]

Part B: Reagents and Biomarker

Standard biomarker used for identification purpose in chromatographic studies was procured from Sigma-Aldrich Private Ltd., Bangalore, India, and solvents from Merck Ltd., Mumbai, India.

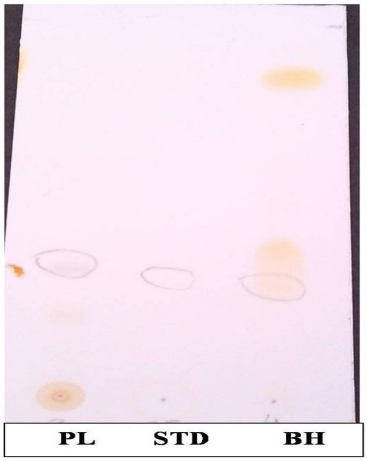


Figure 1: TLC of *Benincasa hispida* seeds and *Polyalthia longifolia* leaves extracts

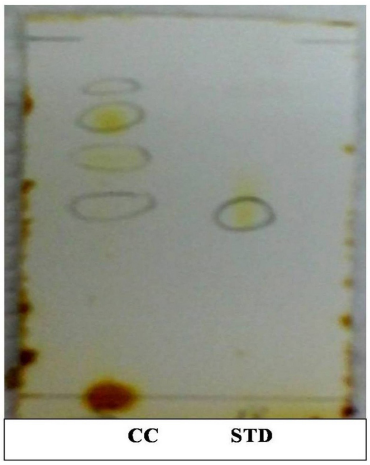


Figure 2: TLC of *Carissa congesta* for β-sitosterol root extract

Part C: Instrumentation

- *Instrument*: CAMAG Linomat 5
- Linomat 5 application parameters
  - *Spray gas*: Inert gas
  - *Sample solvent type*: Ethanol
  - *Dosage speed*: 100 nl/s
  - *Predosage volume*: 0.2 µl
- Sequence
  - *Syringe size*: 100 µl
  - *Number of tracks*: 10
  - *Application position Y*: 12.0 mm
  - *Band length*: 8.0 mm
- Calibration parameters
  - *Calibration mode*: Single level
  - *Statistics mode*: CV
  - *Evaluation mode*: Area and peak height

METHODS

Our research studies enlist the same flow pattern as we have reported in our previous studies for lupeol identification in these extracts. Here, we have estimated percentage of β-sitosterol present as an active constituent in these extracts by Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC).[24-26]

(a) Thin Layer Chromatography (TLC)

Mobile Phase: Hexane: Ethyl Acetate (8.3:1.7-BH and PL) and (7: 3-CC)

Dilution: The standard and the sample were dissolved in ethanol and was filtered using Whatman Filter paper no. 41 before spotting on TLC plate.

Saturation: Chamber was saturated for 30 mins.

(b) High Performance Thin Layer Chromatography (HPTLC)

The HPTLC was performed at Radiant Research Services Private Limited, Bangalore, India. The HPTLC plates (20x10 cm) coated with silica gel 60 F254 were used and scanning of the developed plates was performed at 336 nm (before derivatization) and 550 nm (after derivatization). The standard and the sample were prepared by dissolving 5.16 mg and 47.5 mg in 10 ml of ethanol each. Spots of 3 µg/l, 6 µg/l, 9 µg/l and 12 µg/l were applied on plates per extract.

Percentage of β-sitosterol = sample area × standard dilution × purity × 100/ standard area × sample dilution × 100

RESULTS

(a) Extraction and Preliminary phytochemical analysis

The results of extraction yield and preliminary analysis of the plant extracts were been previously reported by us. [24-26]

(b) Thin Layer Chromatography (TLC)

In TLC, Chromatographic analysis of extracts revealed that Rf value for standard β-sitosterol and the extracts (BH, CC and PL) were found to be 0.72 to 0.77 which confirms the presence of the constituent in these plants (Figures 1 and 2).

(c) High Performance Thin Layer Chromatography (HPTLC)

HPTLC reports interpreted that BH, CC and PL extracts showed well-resolved spots at tracks 5 and 6 in

Table 1: HPTLC analysis of *Benincasa hispida* and standard β-sitosterol

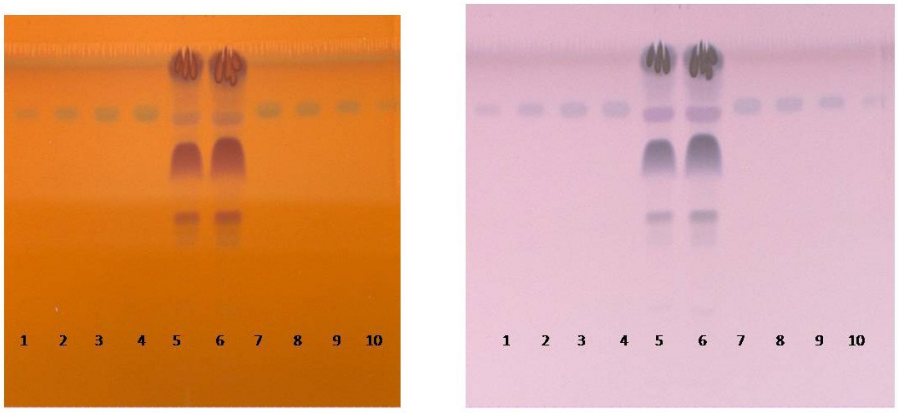
Track No	Details	R_f Value	Area
1	β-sitosterol standard (3 µg/l)	0.74	1297.3
2	β-sitosterol standard (6 µg/l)	0.74	2240.1
3	β-sitosterol standard (9 µg/l)	0.73	2620.9
4	β-sitosterol standard (12 µg/l)	0.73	2784.4
5	*Benincasa hispida* petroleum ether extract (6 µg/l)	0.72	4476.6
6	*Benincasa hispida* petroleum ether extract (9 µg/l)	0.72	4155.6
7	β-sitosterol (12 µg/l)	0.74	2767.4
8	β-sitosterol (9 µg/l)	0.74	2386.8
9	β-sitosterol (6 µg/l)	0.75	1923.0
10	β-sitosterol (3 µg/l)	0.76	891.5

Table 2: HPTLC analysis of *Carissa congesta* roots extract and standard β–sitosterol

Track No	Details	R_f Value	Area
1	β–sitosterol Standard (3 µg/l)	0.75	2215.5
2	β–sitosterol Standard (6 µg/l)	0.75	3383.8
3	β–sitosterol Standard (9 µg/l)	0.74	3924.8
4	β–sitosterol Standard (12 µg/l)	0.73	4634.4
5	*Carissa congesta* petroleum ether extract (6 µg/l)	0.73	2123.1
6	*Carissa congesta* petroleum ether extract (9 µg/l)	0.73	2603.9
7	β–sitosterol Standard (12 µg/l)	0.73	4695.7
8	β–sitosterol Standard (9 µg/l)	0.73	4226.0
9	β–sitosterol Standard (6 µg/l)	0.74	3597.7
10	β–sitosterol Standard (3 µg/l)	0.74	2465.7

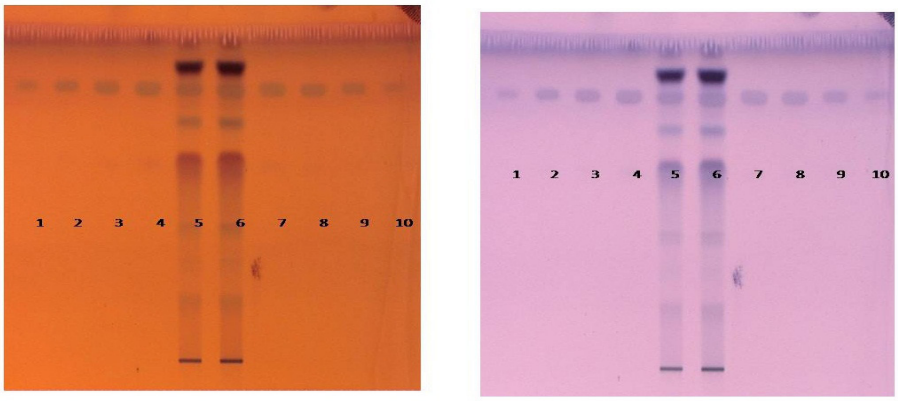
Table 3: HPTLC analysis of *Polyalthia longifolia* leaves extract and standard β–sitosterol

Track No	Details	R_f Value	Area
1	β–sitosterol Standard (3 µg/l)	0.74	2184.2
2	β–sitosterol Standard(6 µg/l)	0.74	2937.1
3	β–sitosterol Standard (9 µg/l)	0.76	3143.2
4	β–sitosterol Standard (12 µg/l)	0.73	3598.5
5	*Polyalthia longifolia* leaves extract (6 µg/l)	0.77	538.9
6	*Polyalthia longifolia* leaves extract (9 µg/l)	0.77	638.7
7	β–sitosterol Standard (12 µg/l)	0.72	3739.8
8	β–sitosterol Standard (9 µg/l)	0.74	3473.7
9	β–sitosterol Standard (6 µg/l)	0.72	2977.6
10	β–sitosterol Standard (3 µg/l)	0.74	2307.4



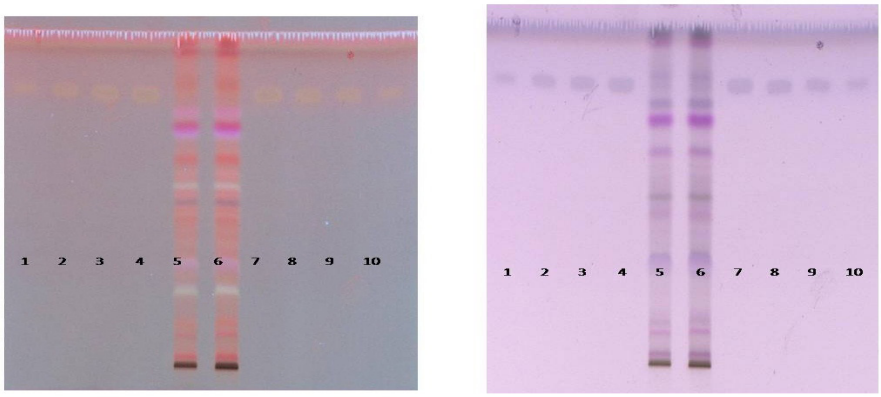
HPTLC Image before derivatization HPTLC Image after derivatization

Figure 3: HPTLC chromatograms of *Benincasa hispida* seeds and standard for β-sitosterol



HPTLC Image before derivatization **HPTLC Image after derivatization**

Figure 4: HPTLC chromatograms of *Carissa congesta* roots and standard for β-sitosterol



HPTLC Image before derivatization **HPTLC Image after derivatization**

Figure 5: HPTLC chromatograms of *Polyalthia longifolia* leaves and standard for β-sitosterol

comparison to the standard at tracks 1, 2, 3, 4, 7, 8, 9 and 10 per extract. The Rf value were found to be equal with standard β-sitosterol [start-0.72, maximum-0.75 and end 0.79 (BH), start–0.73, maximum-0.75 and end-0.79 (CC) and start–0.77, maximum-0.80 and end-0.80 (PL)]. (Table 1, 2, 3, and Figures 3-12, 15-24, 27-36). The amount of β-sitosterol present was 23.00, 5.94, 1.81% w/w (*i.e.* 10.86 (BH), 2.96 mg (CC) mg and 0.89 mg (PL) of β-sitosterol present in 47.5 mg of extracts respectively according to the formula). The images were obtained at 336 nm and 550 nm before and after derivatization respectively releaved the presence of these constituent in the extracts. (Table 1 2 and 3; Figures 3, 4 and 5).

DISCUSSION

A plant extract contains vast plethora of phytoconstituents correlated to their enormous pharmacological activity. Chromatography means "colour writing" which is a useful analytical method of organic and inorganic substances, fractionation of complex mixtures, separation of closely related compounds and in the isolation of unstable substances. Qualitative measurements on plant extracts are determinations of amounts of components present in it. Quantitative measurements involve determination of amount of individual components within a particular class of constituent by gas liquid chromatography or high performance liquid chromatography.[1,27-32]

The technique uses TLC plate coated with fine microparticles of silica is termed as HPTLC. The analytical technique helped us in separating and identification of the active β-sitosterol component from these extracts. HPTLC is sophisticated powerful visualization technique preferred in the detection of the multiple phytoconstituents from herbal extracts due to its reliability, rapidity, surety, reproducibility and linearity.[27] Large number of samples could be analyzed

in a single run simultaneously. The analytical technique selected is specific and solution stable, provides linearity of 0.999 and 99.24% of sample.[1,33-35] Thus, the results depicted in TLC and HPTLC experimentation played a vital role in our research helping us to identify the β-sitosterol with their percentage in the plant extracts.

CONCLUSION

β-sitosterol has been noticed to play an important role in various plant extracts signifying its pharmacotherapeutic potential. Our research studies directs the field of the pharmacognosist budding professionals towards the identified constituent in the these extracts of BH, CC and PL which were confirmed by simple methods like TLC and HPTLC. The current research studies paves the pathway for research thrust scientists to consider the other probable identified constituents in the extracts and correlate there utilities on pharmacological models of different therapeutic categories.

CONFLICT OF INTEREST

We declare no conflict of Interest.

ACKNOWLEDGEMENTS

We would like to acknowledge the college management who provided us all the facilities to do this work as well as Radiant Research Services Pvt. Ltd. for their help in analysis.

Highlights of Paper

- Fruits of *Benincasa hispida* (BH) is regarded as Valliphala , *Carissa congesta* roots (CC) are important in rural communities and *Polyalthia longifolia leaves* (PL) is an ornamentally flowering plant in India system.
- In the recent studies, the research team determined the percentage of the β-sitosterol present in the BH, CC and PL by High Performance Thin Layer Chromatography.
- The amount of β-sitosterol present in the BH seeds, CC roots and PL leaves extracts was found to be 23.00, 5.94 and 1.81 % w/w respectively. The extracts showed peak that coincided with standard peak of β-sitosterol.

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Hepatoprotective Activity of *Michelia nilagirica* against Paracetamol Induced Hepatic Injury in Rats

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ABSTRACT

Background: *Michelia nilagirica* belonging to the family Mangoliaceae is commonly used by many traditional healers in most of the herbal preparations for diabetes and kidney diseases. **Objective:** Different fractions isolated from ethanolic extract of whole plant of *Michelia nilagirica* is investigated for hepatoprotective activity in wistar albino rats against paracetamol induced hepatic injury. **Materials & Methods:** Rats were divided into eight groups. Each group contains six animals. Hepatic injury was achieved by injecting paracetamol at a dose of 2 mg/kg p.o. **Results:** The hepatoprotective action is seen with fraction A by reduction in serum marker enzymes like Aspartate transaminase (AST), Alanine transaminase (ALT). It also reduced the elevated levels of Alkaline phosphotase (ALP) & Serum bilirubin. **Conclusion:** Histopathological studies further confined the hepatoprotective activity of fraction A against paracetamol treated group. The results obtained were compared with silymarin (100 mg/kg, orally), a standard drug.

Key words: Albino rats, Hepatoprotective, *Michelia nilagirica*, Paracetamol, Screening.

INTRODUCTION

The liver is a important organ of vertebrates and some other animals and some other animals. It has a wide range of functions like ability to detoxicate toxic substances,[1] synthesis of proteins, production of biochemicals necessary for digestion. Hepatocytes (Liver cells) regulates a variety of biochemical reactions like synthesis and break down of small and complex molecules and many other vital functions. Liver diseases are mainly due to infections, life style (smoking, alcoholism), toxic substances (chemicals & drugs). Hepatotoxic agents damage liver cells by lipid peroxidation and other oxidative damages. In addition, many biochemical markers in the serum like aspartate transaminase[2] (AST), alanine transaminase (ALT), alkaline phosphatase[3] (ALP) and bilirubin[4] were also increased. In absence of a reliable liver protective drug in the modern system of medicine, a number of novel medicinal preparations in Ayurveda, Homeopathy, Unani, the Indian system of medicine are recommended for the treatment of liver disorders. So medicinal plants are considered to be effective and safe natural remedy treatments for hepatotoxicity.[5]

Michelia nilagirica, belonging to the genus magnolia (magnoliaceae) is a native to tropical and subtropical South and Southeast Asia, including southern china. It is widely used in both Ayurveda and Homeopathic medicine. Flower buds of *Michelia champaca* Linn. is commonly used by many traditional healers in most of the herbal preparations for diabetes[6] and kidney diseases.[7] Traditionally, it is being used in fever, colic, leprosy, post partum protection[8] and in eye disorders.[9] It has been reported to possess antipyretic, antiulcer, anti-inflammatory,[10] insecticidal,[11] antioxidant, antimicrobial[8] and leishmanicidal[12] activities. The active constituents reported in this plant are alkaloids, tannins, saponins, sterols, flavonoids and triterpenoids.[8] In Ayurveda, traditional usages of plants are most commonly in their aqueous extracts form only. Concurrently, some of the papers searched focus for testing these plants in their ethanolic or aqueous extracts and some have also reported activity in petroleum ether, benzene and chloroform

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extracts.[13-15] Keeping these facts in view, the present study was undertaken to create a scientific base for the use of the extract of *Michelia nilagirica* as a hepatoprotective agent.

Paracetamol is a commonly used analgesic & antipyretic agent. The main adverse effects of paracetamol overdose are hepatotoxicity & nephrotoxicity which could be fatal. Its metabolite N-acetyl-p-benzoquinoneimine (NAPQI) which is normally detoxified by glutathione is responsible for hepatotoxicity. Excess of NAPQI is formed in paracetamol overdose which binds to proteins and other macromolecules resulting in hepatic necrosis. The standard therapy for paracetamol overdose is N-acetylcysteine. It restores hepatic glutathione which detoxifies NAQPI.[16] Some cases do not respond,[17] though N-acetylcysteine is used for the treatment of paracetamol toxicity. In spite of tremendous advances in modem medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatocytes. To treat a wide variety of clinical diseases including liver disease many active plant extracts are frequently utilized. Therefore, searching for effective and safe drugs for liver disorders are continues to be an area of interest.[18]

MATERIALS AND METHODS

Collection of plant material

The whole plant of *Michelia nilagirica* was collected from the deciduous forest of Tirumala Hills in Andhra Pradesh State, India. Samples were authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, India. The whole plant of *Michelia nilagirica* were sorted, cleaned and air-dried at room temperature for one week. By using the laboratory hammer mill these were ground to powder. Powdered samples were collected and stored in air- and water-proof containers protected from direct sunlight and heat until required for extraction.

Preparation of extracts

The powdered materials of *Michelia nilagirica* (whole plant) were extracted successively each for 18 hrs with petroleum ether, ethyl acetate, chloroform, ethanol and distilled water in soxhlet apparatus. The extracts were concentrated to dryness in rota evaporator till free from the solvents.

Isolation of fractions

Thin-layer chromatography method was carried out using silica gel aluminum plate 60F-254, 0.5 mm (TLC plates, Merck). The spots were visualized in UV light and 10% of H_2SO_4 in methanol. The ethanolic extract was subjected to column chromatography (silica gel 60-100) for further purification. The equilibration of column was carried for one hour with petroleum ether at flow rate 5ml/min. The sample was (2 gm dissolve in acetone) loaded on to the column, 8 fractions were collected using petroleum ether:ethyl acetate (4:1), petroleum ether:ethyl acetate (1:1), petroleum ether:ethyl acetate (2:3), ethyl acetate (100%), chloroform:methanol (9:1), chloroform:methanol (1:1) and chloroform:methanol (2:8).

Above yielded product were pooled into five fractions based on TLC. The yield and appearance of the five fractions was fraction A 50 mg/gm & yellow, fraction B 300 mg/gm & black, fraction C 150 mg/gm & green, fraction D 200 mg/gm & darkish brown and fraction E 150 mg/gm & saffron.

Phytochemical analysis

Phytochemical analysis[19] of fractions was carried out for the presence of alkaloids, tannins, saponins, glycosides, terpenoids, carbohydrates, flavonoids, proteins, amino acids, fixed oils, steroids & sterols by different methods.

Experimental design

Albino rats of wistar strain weighing 150-200 gm were purchased from National Institute of Nutrition, Hyderabad. The rats were kept in polypropylene cages (3 in each cage) at an ambient temperature of 25 ± 2°C and relative humidity of 55–65%. A 12 hrs light and dark schedule was maintained in the air conditioned animal house. All the rats were fed with common diets for week after arrival and then divided into groups with free access to food and water.

Acute toxicity studies

Toxicological studies were conducted in 3 groups of 5 mice and the extract is administered at the doses of 150, 300 and 1000 mg/kg no mortality was observed. They were observed continuously for 1 hr for any gross behavioral changes, symptoms of toxicity and mortality if any after dosing with *Michelia nilagirica* ethanol extract.

Study design

Rats were divided into 6 groups of six animals in each.[20]

Group I : Received Tween 80

Group II : Paracetamol (2 gm/kg)

Group III : Silymarin (100 mg/kg)

Group IV : Fraction A (70 mg/kg)

Group V : Fraction B (70 mg/kg)

Group VI : Fraction C (70 mg/kg)

Group VII : Fraction D (70 mg/kg)

Group VIII : Fraction E (70 mg/kg)

Given orally the following treatment for seven days. On the seventh day, paracetamol suspension was administered at a dose of 2 gm/kg body wt., p.o., to rats of groups III, IV, V, VI, VII and VIII.

Biochemical estimation

After 24 hrs of the last treatment, blood was collected from retro-orbital plexus, serum was separated by centrifugation at 10000 rpm for 15 min. The serum was then collected and analyzed for various biochemical parameters like Aspartate transaminase (AST), Alanine transaminase[21] (ALT), Alkaline phosphatase[22] (ALP) and bilirubi[23]

Histopathological studies

The liver tissue was collected, fixed in 10% formalin and stained with hematoxylin and eosin for photomicroscopic observation.[24]

Statistical analysis

The data was represented as Mean±SD. Results were analysed by One-Way ANOVA followed by Dunnett's multiple comparison test using SPSS software. The minimum level of significance was set at $p<0.05$.

RESULTS

Preliminary phytochemical screening

Phytochemical screening revealed the presence of flavonoids & amino acids in fraction A, terpenoids & proteins in fraction B, terpenoids in fraction D and alkaloids, tannins, carbohydrates & flavonoids in fraction E.

Biochemical parameters

Acute paracetamol administration significantly increased the level of liver injury marker enzymes like AST, ALT, ALP and bilirubin (The liver marker enzymes were 240 ± 2.8 U/L, 185.5 ± 9.8 U/L, 647.8 ± 39.5 U/L and 2.18 ± 0.31 mg/dl for the SGOT, SGPT, ALP and bilirubin respectively). Pretreatment with fraction A (70 mg/kg) and fraction B (70 mg/kg for seven days) reduced raising serum enzymes in hepatotoxic rats (The liver marker enzymes were 155.5 ± 15.5, 121 ± 3.53, 427.5 ± 14.8 & 1.15 ± 0.23 for fraction A and 176.8 ± 13.4, 122.3 ± 10.6, 497 ± 7.07 & 1.56 ± 0.45 for fraction B for the biochemical parameters the AST, ALT, ALP & bilirubin respectively). Pretreatment with fraction C, D and E (70 mg/kg) resulted no significant changes in Serum AST, ALT, ALP & bilirubin levels compare to paracetamol induced hepatotoxic rats (Figures 1-5).

Histopathological examination

Photographs showing histopathology of liver sections (5-10 µ), 40X, Haematoxylin-eosin stain. A) Control group (tween 80) showed normal histology, B) Paracetamol (2 gm/kg) treated group showed cellular degeneration, hydropic changes, fatty changes with wide spread hepatocellular necrosis, C) Silymarin (100 mg/kg) + paracetamol (2 mg/kg) treated group showed very little necrosis or degeneration, D) Fraction A (7 0 mg/kg) + paracetamol

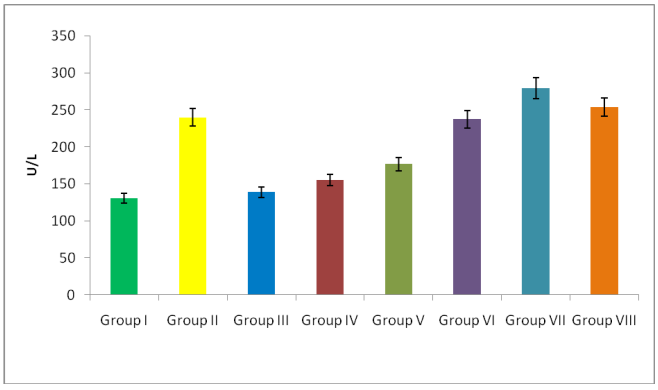

Figure 1: Effect of *Michelia nilagirica* on rat serum (AST) after paracetamol administration

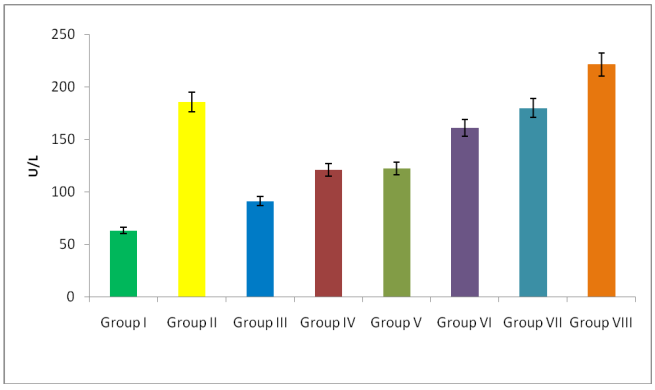

Figure 2: Effect of *Michelia nilagirica* on rat serum (ALP) after paracetamol administration

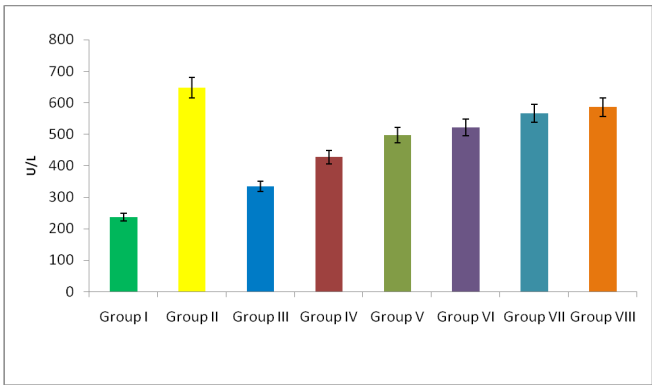

Figure 3: Effect of *Michelia nilagirica* on rat serum (ALT) after paracetamol administration

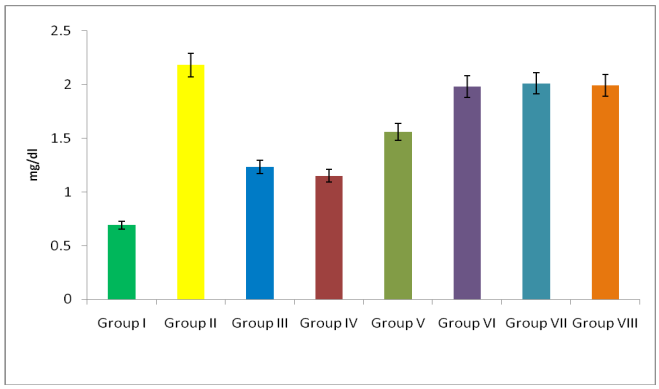

Figure 4: Effect of *Michelia nilagirica* on rat Serum bilirubin after paracetamol administration

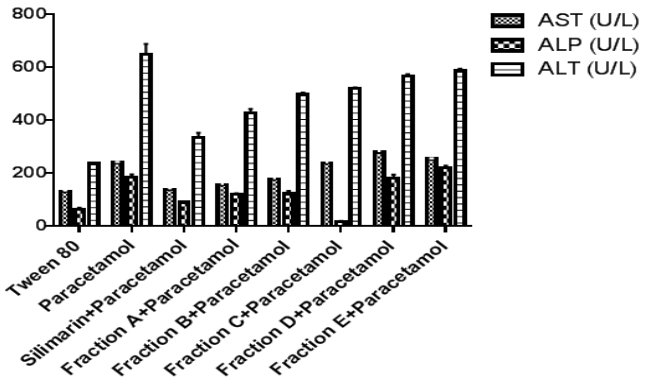

Figure 5: Comparison of AST, ALP & ALT values of different fractions

(2 mg/kg) treated group showed no hepatocellular damage except areas of focal degeneration and sinusoidal dilation, E) Fraction B (70 mg/kg) + paracetamol (2 mg/kg) treated group showed minimal hepatocellular damage, fatty changes and loss of liver architecture, F) Fraction C extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatic necrosis, cellular degeneration, hydropic changes and fatty changes, G) Fraction D extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatocellular damage and fatty changes, H) Fraction E extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatic necrosis, cellular degeneration, hydropic changes and fatty changes (Figure 6).

DISCUSSION

Paracetamol is a common analgesic and antipyretic drug. Several studies have demonstrated the induction of hepatocellular damage or necrosis by paracetamol higher doses in experimental animals and humans.[25] For screening of hepatoprotective agents, paracetamol-induced hepatotoxicity has been used as a reliable method. Paracetamol is metabolized primarily in the liver and eliminated by conjugation with sulfate and glucuronide, and then excreted by the kidney. Moreover, paracetamol hepatotoxicity has been attributed to the formation of toxic metabolites, when a part of paracetamol is activated by hepatic cytochrome P-450 to a highly reactive metabolite Nacetyl-p-benzoquinoneimine[26] (NAPQI). Toxic metabolites (N-acetyl-p-benzoquineimine) can alkylate and oxidise intracellular GSH, which results in liver GSH depletion subsequently leads to increased lipid peroxidation by abstracting hydrogen from a polyunsaturated fatty acid and ultimately, liver damage due to higher doses of paracetamol.[27,28] Reactive metabolites can exert initial cell stress through a wide range of mechanisms including depletion of glutathione (GSH) or binding to enzymes, lipids, nucleic acids and other cell structures.[29]

Acute paracetamol administration significantly increased the level of liver injury marker enzymes like AST, ALP, ALP and bilirubin (The liver marker enzymes were 240 ± 2.8 U/L, 185.5 ± 9.8 U/L, 647.8 ± 39.5 U/L and

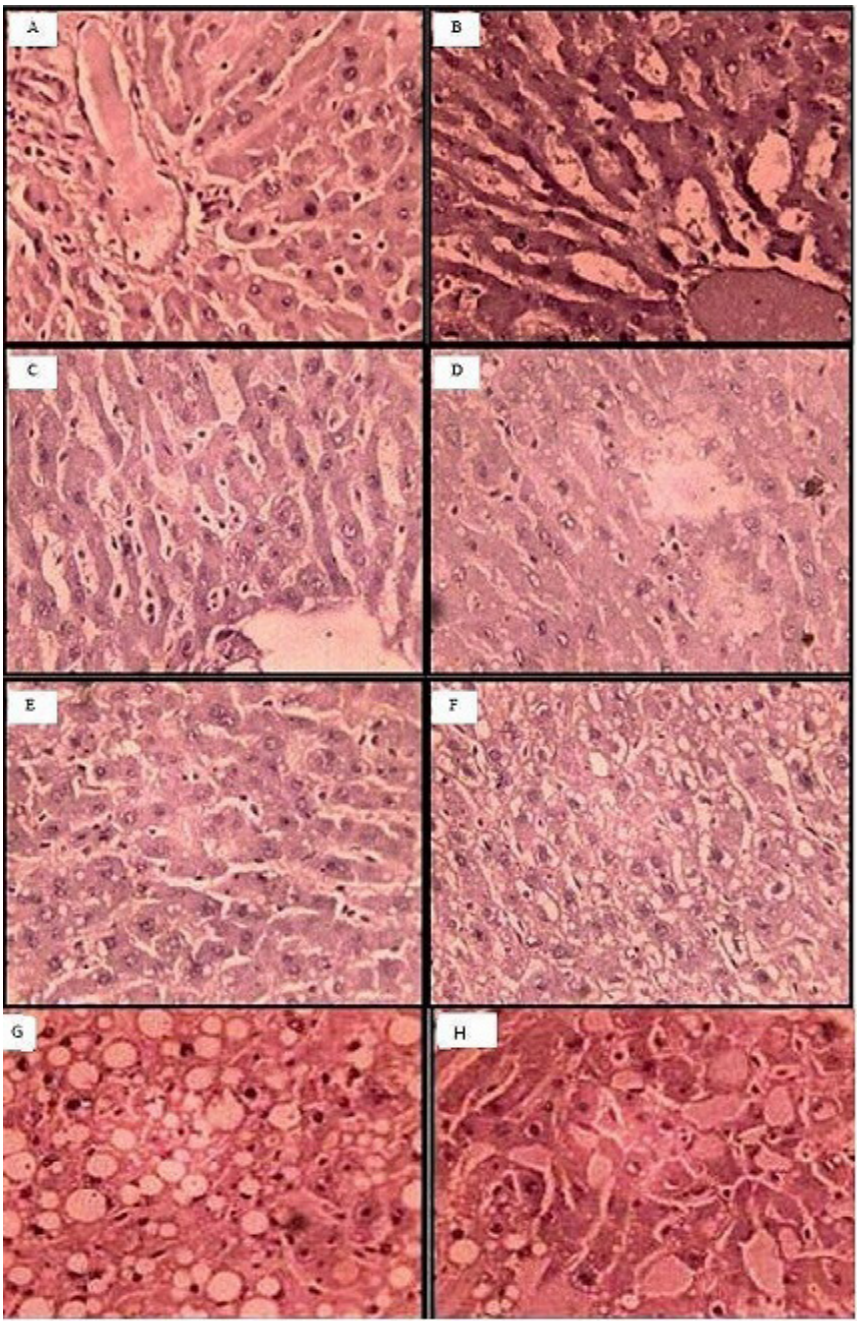

Figure 6: Histopathological section of liver tissue of albino rats

A) Control group (tween 80) showed normal histology, B) Paracetamol (2 gm/kg) treated group showed cellular degeneration, hydropic changes, fatty changes with wide spread hepatocellular necrosis, C) Silymarin (100 mg/kg) + paracetamol (2 mg/kg) treated group showed very little necrosis or degeneration, D) Fraction A (70 mg/kg) + paracetamol (2 mg/kg) treated group showed no hepatocellular damage except areas of focal degeneration and sinusoidal dilation, E) Fraction B (70 mg/kg) + paracetamol (2 mg/kg) treated group showed minimal hepatocellular damage, fatty changes and loss of liver architecture, F) Fraction C extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatic necrosis, cellular degeneration, hydropic changes and fatty changes, G) Fraction D extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatocellular damage and fatty changes, H) Fraction E extract (70 mg/kg) + paracetamol (2 mg/kg) treated group showed hepatic necrosis, cellular degeneration, hydropic changes and fatty changes.

2.18 ± 0.31 mg/dl for the AST, ALP, ALP and bilirubin respectively). A similar experimental procedure also used by other researchers to report the hepatoprotective effect of natural products.[30]

The present study reported the degree of protection, measured by using biochemical parameters like AST, ALT, ALP and bilirubin in fractions treated rats.

Studies showed that the pretreatment with *Michelia nilagirica* fraction A (70 mg/kg) for seven days offers considerable protection ($P<0.05$) to liver as evidenced from the levels of biochemical parameters (The liver marker enzymes were 155.5 ± 15.5, 121 ± 3.53, 427.5 ± 14.8 & 1.15 ± 0.23 for

Table 1: Phytochemical screening of the various fractions

Phytochemicals	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
Alkaloids	----	----	----	----	+
Tanins	----	----	----	----	+
Saponins	----	----	----	----	----
Glycosides	----	----	----	----	----
Terpinoids	----	+	----	+	----
Carbohydrates	----	----	----	----	+
Flavonoids	+	----	----	----	+
Proteins	----	+	----	----	----
Aminoacids	+	----	----	----	----
Fixed oils	----	----	----	----	----
Steroids & Sterols	----	----	----	----	----

Table 2: Effect of *Michelia nilagirica* on the serum AST, ALP, ALT & bilirubin levels on albino rats

Group	Treatment	AST (U/L)		ALP (U/L)		ALT (U/L)		Bilirubin (mg/dl)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
I	Tween 80	131.1	5.6	63.2	6.4	236	4.24	0.69	0.03
II	Paracetamol (2 gm/kg)	240	2.82	185.5	9.89	647.8	39.59	2.18	0.31
III	Sylimarine (100 mg/kg) + Paracetamol (2 gm/kg)	138.8	7.77	91.3	1.41	333.5	19.09	1.23	0.2
IV	Fraction A (70 mg/kg) + Paracetamol (2 gm/kg)	155.5	15.5	121	3.53	427.5	14.8	1.15	0.23
V	Fraction B (70 mg/kg) + Paracetamol (2 gm/kg)	176.8	13.4	122.3	10.6	497.3	7.07	1.56	0.45
VI	Fraction C (70 mg/kg) + Paracetamol (2 gm/kg)	237.6	16.9	160.8	2.9	521	2.8	1.98	0.33
VII	Fraction D (70 mg/kg) + Paracetamol (2 gm/kg)	279.5	28.2	180	14.8	566.5	7.01	2.01	0.58
VIII	Fraction E (70 mg/kg) + Paracetamol (2 gm/kg)	254	2.51	221.5	7.05	586.1	8.48	1.99	0.46

the AST, ALT, ALP and bilirubin respectively) and similarly fraction B (70 mg/kg) significantly ($P<0.05$) reduced raising serum enzymes in hepatotoxic rats (Table 1). Pretreatment with fraction D and E (70 mg/kg) resulted no significant reduction in serum AST, ALT, ALP and bilirubin compared to fraction A and B treated groups (Table 2).

CONCLUSION

Flavonoids[31] are a class of secondary plant phenolics found in fruits, vegetables and foods, which act as pharmacological active compound in many medicinal plants. Many of the biological action of flavonoids[32] have been attributed their powerful antioxidant properties[33] and number of scientific reports indicated that certain flavonoids, terpenoids have protective effect on liver due to its antioxidant properties.[34]

Present study fraction A contain valuable flavonoids, that shown hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress by their antioxidant property.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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Highlights of Paper

- *Michelia nilagirica* belonging to the family Mangoliaceae is commonly used by many traditional healers in most of the herbal preparations for diabetes and kidney diseases.
- Different fractions isolated from ethanolic extract of whole plant of *Michelia nilagirica* is investigated for hepatoprotective activity in wistar albino rats against paracetamol induced hepatic injury.
- Rats were divided into eight groups. Each group contains six animals. Hepatic injury was achieved by injecting paracetamol at a dose of 2 mg/kg p.o.
- The hepatoprotective action is seen with fraction A by reduction in serum marker enzymes like aspartate transaminase (AST), alanine transaminase (ALT). It also reduced the elevated levels of alkaline phosphotase (ALP) & serum bilirubin.
- Histopathological studies further confined the hepatoprotective activity of fraction A against paracetamol treated group. The results obtained were compared with silymarin (100 mg/kg, orally), a standard drug.

Author Profile

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- **Dr. Atmakuri Lakshmana Rao:** He is having 15 years of Teaching and Research experience and serving as Professor and Principal of V. V. Institute of Pharmaceutical Sciences, Gudlavalleru. He guided 03 Ph.D. candidates and 18 M.Pharm. Candidates. He conferred with Meritorious University Best Teacher Award from Jawaharlal Nehru Technological University Kakinada, Kakinada. He is ratified as Principal by Jawaharlal Nehru Technological University Kakinada, Kakinada and ratified as Associate Professor by Andhra University, Visakhapatnam. He received Dr. P.D. Sethi Annual Award Certificate of Merit from Anchrom Enterprises (I) Pvt. Ltd., Mumbai. He is Faculty Accreditation and Evaluation Member, International Accreditation Organization, USA. He is given 5 Guest Lectures in different universities. He published 158 papers in various International and National Journals. He published 2 e-Books. He is Editor-in-Chief, Reviewer and Editorial Board Member for 40 reputed International and National journals. He organized 11 Seminars, Conferences and Workshops. He participated in 62 Seminars, Conferences and Workshops. He presented 27 scientific papers in various Seminars and Conferences and received 09 prizes. He is having memberships in 10 Professional Societies.

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Anxiolytic Potential of Methanol Extract from *Ageratum conyzoides* Linn Leaves

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ABSTRACT

Objective: *Ageratum conyzoides* Linn. (Asteraceae) has been widely used in African traditional medicine for healing mental and infectious diseases. The present study was designed to scientifically validate the traditional claim of *A. conzyoides* as anti-anxiety drug and to identify the compound responsible for the anxiolytic effects of *A. conzyoides*. **Method:** The methanol extract of *A. conzyoides* was prepared by soxhlet apparatus. The methanol extract was fractionated into ethylacetate and butanol fractions by liquid-liquid partitioning method. Methanol extract (100 and 200 mg/kg; p.o.) and its prepared fractions (25 and 50 mg/kg; p.o.) were evaluated for anxiolytic activity in mice by using elevated plus maze (EPM) model. Thin layer chromatography studies were performed to identify the possible anxiolytic component. **Results:** Methanol extract at both doses showed significant, when compared to vehicle control group, increase in time spent and number of entries in open arms of EPM confirming the anti-anxiety effects of *A. conzyoides*. Liquid-liquid partitioning of methanol extract gave two fractions (ethylacetate and butanol) which were administrated at 25 and 50 mg/kg doses to mice in EPM, respectively. Results showed that ethylacetate fraction was responsible for anxiolytic effects of methanol extract of *A. conzyoides*. The TLC studies were carried out for ethylacetate fraction and Quercetin was identified by comparing R_f values with the standard (Quercetin). **Conclusion:** The present investigation revealed that the extract has significant anxiolytic effect. The flavonoid quercetin may be responsible for the observed anxiolytic effects of *A. conyzoides*.

Key words: *Ageratum conyzoides*, Anxiolytic, Methanol extract, Quercetin, TLC.

INTRODUCTION

Anxiety disorders, nowadays, are one of the common causes for frailty. Nearly one fourth of the adult population suffers from these psychiatric disorders during the course of their life. Women are more prone to anxiety disorders (30.5%) as compared to men (19.2%). Research data revealed that very few people (<14%) suffering from such disorders receive treatment.[1] Due to worldwide increase in the incidence of anxiety disorders, reaching about 16.6%, advancements have been made regarding their causes and treatments.[2] Cognition, psychomotor function and intellect, all are hampered by anxiety. Benzodiazepines are considered top notch among all the existing drug treatments for anxiety.[3] But there are numerous side effects linked with the present treatment of anxiety such as cardiotoxicity, hypertension, blood dyscrasia, impairment of memory and attention.[4] The past era has encouraged the practice of complementary and alternative medicine. The ability of herbal drugs in recuperating various behavioral disorders has been bolstered by plentiful of research investigations.[5-7]

Ageratum conyzoides is an annual branching herb belonging to family Asteraceae with nearly 90 cm height.[8] The plant is commonly known as Goatweed, Floss flower or Whiteweed.[9] It has been used customarily in certain African realms for the treatment of psychological and contagious ailments.[10] Aerial parts of the plant are used to cure diabetes.[11] Plant foliage is used to heal cuts and injuries as well as an antidote for snake bite.[12] Leaves are also used for blood coagulation.[13] Reports reveal that the

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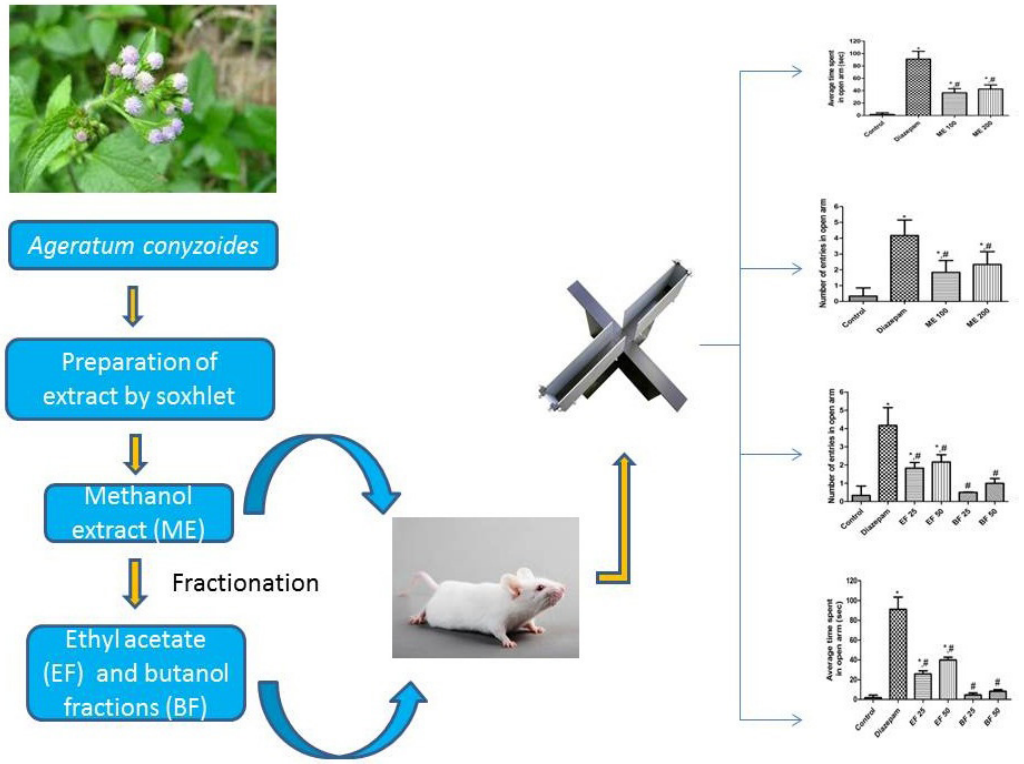

Graphical Abstract

leaves of the plant act as mines full of flavonoids namely quercetin, quercetin-3-rhamnopiranoside, kaempferol, kaempferol-3-rhamnopiranoside, 5'-methoxynobiletin, linderoflavone B.[10] There are traditional claims for the root of the plant being used as anthelmintic and antidysentric.[14] Recently, Varadharajan and Rajalingam, (2011) reported the antiepileptic potential of *A.conyzoides* in rats.[15]

Hence, the present study has been designed to scientifically validate the traditional claim of *A. conyzoides* as antianxiety agent.

MATERIALS AND METHODS

Plant material

Leaves of *Ageratum conyzoides* were collected from local areas around the Guru Nanak Dev University, Amritsar, Punjab, India. The taxonomic identity of plant was confirmed by Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India. A voucher specimen no S.R. Bot Sci/97 herb has been deposited in the herbarium of Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India. Leaves were dried under shade and milled in grinder to fine powder.

Preparation of Extracts

The powdered leaves (500 g) were subjected to successive Soxhlet extraction for 48 hours by using solvents in increasing polarity viz. petroleum ether (60-80°C) and methanol. Each extract was concentrated by distilling off the solvent using rotavapour and then evaporated to dryness on the water-bath. Extracts were weighed and percentage yield was calculated in terms of the air-dried weight of the plant material.

Fractionation of the methanol extract

Twenty two grams of the methanol extract was fractionated by dissolving in 100 mL of distilled water. Then extraction was done with hexane (600 ml) and aqueous phase was collected. After that successive liquid extraction of aqueous phase was done with ethyl acetate (3 X 150 ml) and butanol (3 X 150 ml). Ethyl acetate fraction of yellow color and butanol fraction of red color were concentrated using a rotary evaporator.

Phytochemical screening

All extracts and fractions were subjected to phytochemical screening to detect the presence of various phytochemicals viz. alkaloids, flavonoids, tannins, anthocyanins and saponins.[16,17]

Drugs

Diazepam (DZP, 2.0 mg/kg, Sigma, India) was used as the standard anxiolytic drug (positive control group). Carboxymethylcellulose (CMC, 1% w/v, Merck, India) was employed to treat negative control group. All other solvents and reagents were of analytical grade.

Animals and treatments

Swiss Albino mice weighing 25-30 g were procured from Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. Eight animals per cage were held and maintained under laboratory conditions at 25^{0}C with a normal 12 h light/dark schedule and free access to water and food. Mice were allowed to acclimatize to the laboratory environment (for at least 3 weeks) prior to the commencement of experimentation. Six mice per group were used in all sets of experiments. Experiments were carried out in a noise free area with controlled lighting, between 8:00 a.m. and 12:00 p.m. All protocols and experiments were conducted in strict compliance according to the ethical principles and guidelines provided by Institutional Animal Ethical Committee (IAEC). The conditions required to obtain reliable data i.e. least number of animals and time of surveillance, were utilized.

The anxiolytic activity of methanol extract and its fractions (ethylacetate and butanol) were evaluated by employing EPM. All the test and standard drugs were administered to mice 30 min prior to experiment via oral and intraperitoneal route respectively. Various animal groups employed in the present study were as follows:

Group I–Vehicle treated: CMC (1 % w/v) was administered orally

Group II and III–Methanol extract (100 and 200 mg/kg)

Group IV and V–Ethylacetate fraction of methanol extract (25 and 50 mg/kg)

Group VI and VII–butanol fraction of methanol extract (25 and 50 mg/kg)

Group VIII–Positive control: diazepam (2 mg/kg)

Elevated plus-maze (EPM)

Selection of drugs that help in modifying anxiety is done with the help of EPM, the most extensively employed model in understanding the psychological and neurochemical basis of anxiety.[18] The EPM model used in the study consisted of two open (30 x 5 x 0.2 cm) and two closed (30 x 5 x 15 cm) arms, stretching out from a platform (5 x 5 cm) in the midway and raised to a height of 45 cm above the ground.[19]

Each animal was positioned at the center of the maze facing one of the enclosed arms, thirty minutes after administration of drugs. Number of entries and time spent in closed and open arms were recorded for 10 min. Entry into an arm was stated as the animal placing all four paws on the arm. The maze was cleaned properly with 5% alcohol, each time before placing the animal, to abolish the possible prejudice due to the scent left by the preceding animal.

Statistical analysis

All data are expressed as mean ± standard deviation (SD) and analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's Multiple Range test using Graph Pad Prism 5.0 software. A p value of <0.05 was considered statistically significant.

RESULTS

Percentage yield and phytochemical screening of prepared extracts and fractions

The methanol extract gave the yield of 10% w/w and its ethylacetate and butanol fractions gave 0.6% w/w and 0.4 % w/w, respectively. Preliminary phytochemical screening of methanol extract revealed the presence of numerous phytochemical groups (Table 1). Both the fractions of methanol extract showed the presence of flavonoids.

Evaluation of anxiolytic activity of methanol extract and its fractions

The methanol extract of *A. conyzoides* showed significant increase in average time spent and mean of number of entries by mice in open arms when compared to control group (Figure 1 and 2) confirming the traditional claim of plant. However, the observed anxiolytic effect at dose of 100 mg/kg of methanol extract was not significantly different when compared to anxiolytic effect of same extract at dose of 200 mg/kg. Administration of diazepam (2 mg/kg) to mice significantly ($p<0.05$) increased the mean time spent by mice in open arms and the average number of entries into the open arms of EPM, confirming an anxiolytic effect.

Among the prepared fractions of methanol extract, only ethylacetate fraction (25 and 50 mg/kg) showed significant ($p<0.05$) anti-anxiety activity which was confirmed by increase in average time spent and mean of number of

Table 1: Phytochemical screening of methanol extract and its fractions

Phytoconstituent	Methanol extract	Ethyl acetate fraction	Butanol fraction
Alkaloids	+	–	–
Carbohydrates	+	–	–
Protein and amino acids	+	–	–
Phytosterols	+	–	–
Flavonoids	+	+	+
Terpenoids	+	–	–
Tannins	+	+	+

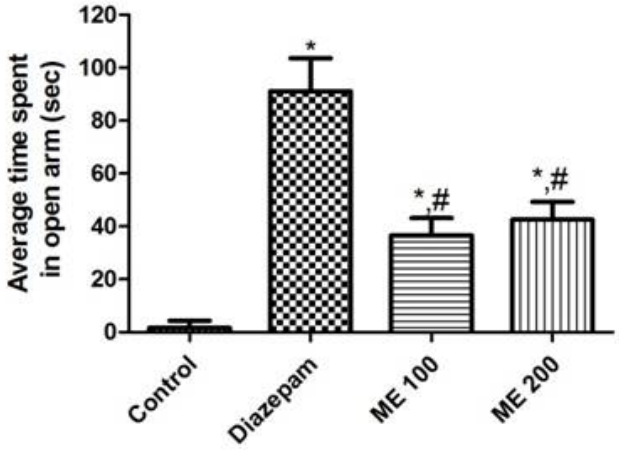

Figure 1: Effect of methanol extract of *Ageratum conyzoides* on average time spent in the open arms by mice exposed to the EPM test

Data are expressed as mean ± S.D; n=6; Anova followed by Tukey'*spost hoc* multiple range test; *p<0.05 versus control; #p<0.05 versus Diazepam; ME 100=methanol extract 100 mg/kg; ME 200=methanol extract 200 mg/kg.

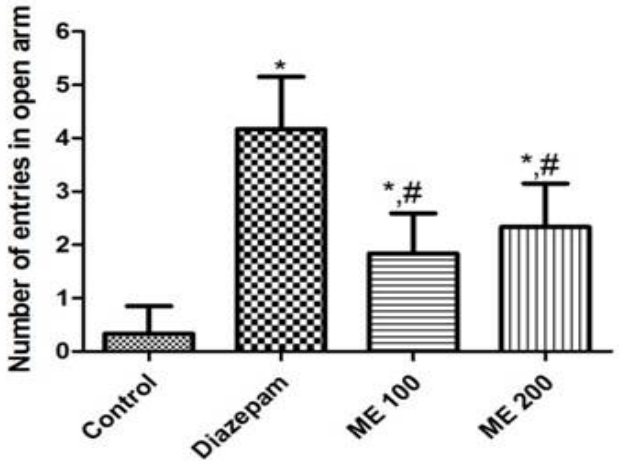

Figure 2: Effect of methanol extract of *Ageratum conyzoides* on number of entries in open arms by mice exposed to the EPM test

Data are expressed as mean ± S.D; n=6; Anova followed by Tukey'*spost hoc* multiple range test; *p<0.05 versus control; #p<0.05 versus Diazepam; ME 100=methanol extract 100 mg/kg; ME 200=methanol extract 200 mg/kg.

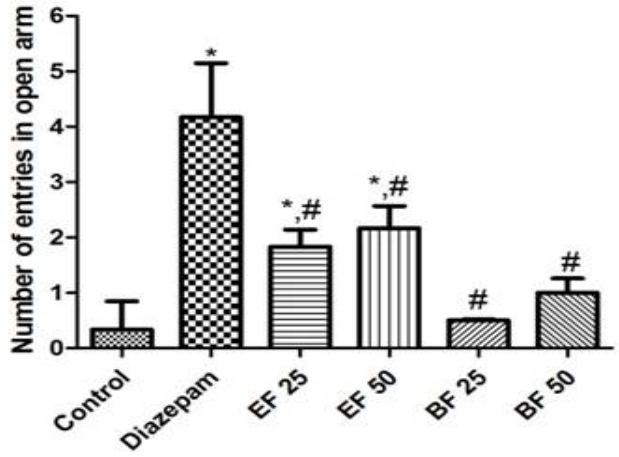

Figure 3: Effect of ethyl acetate and butanol fractions of methanol extract of *Ageratum conyzoides* on average time spent in the open arms by mice exposed to the EPM test

Data are expressed as mean ± S.D; n=6; Anova followed by Tukey'*spost hoc* multiple range test; *p<0.05 versus control; #p<0.05 versus Diazepam; EF 25 = ethyl acetate fraction 25 mg/kg; EF 50=ethyl acetate fraction 50 mg/kg; BF 25=butanol fraction 25 mg/kg; BF=butanol fraction 50 mg/kg.

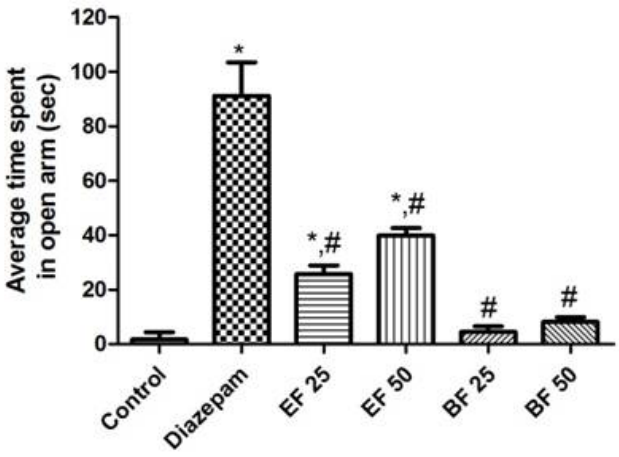

Figure 4: Effect of ethyl acetate and butanol fractions of methanol extract of *Ageratum conyzoides* on number of entries in open arms by mice exposed to the EPM test

Data are expressed as mean ± S.D; n=6; Anova followed by Tukey'*spost hoc* multiple range test; *p < 0.05 versus control; #p<0.05 versus Diazepam; EF 25=ethyl acetate fraction 25 mg/kg; EF 50=ethyl acetate fraction 50 mg/kg; BF 25=butanol fraction 25 mg/kg; BF=butanol fraction 50 mg/kg.

Figure 5: Comparison of TLC profile of Quercetin (left side) and ethylacetate fraction (right side) of methanol extract

entries by mice in open arms of EPM when compared to control group. The butanol fraction of methanol extract of *A. conyzoides* did not show any significant anti-anxiety activity (Figure 3 and 4).

Thin layer chromatography study of ethylacetate fraction of methanol extract

TLC chromatogram of ethylacetate fraction of methanol extract and standard flavonoid i.e. quercetin was developed by using toluene: ethylacetate: formic acid in ratio of 10:3:1 v/v/v as mobile phase. The developed chromatogram of ethylacetate fraction of methanol extract showed the presence of quercetin. This was confirmed by comparing R_f values of ethylacetate fraction with standard quercetin (Figure 5).

DISCUSSION

Ageratum conyzoides is a plant species that is broadly employed as a remedy for mental disorders. In the present study, *Ageratum conyzoides* was evaluated for anxiolytic effect in order to scientifically validate the traditional claim by using behavioral models namely elevated plus maze. The oral administration of methanol extract (100 mg/kg and 200 mg/kg)to mice showed anti-anxiety effects indicated by increase in average time spent and number of entries in open arm of EPM. Phytochemical screening of methanol extract showed the presence of flavonoids along with other phytochemical groups. The chemical separation of the active extract allowed isolation of an active fraction (ethylacetate fraction) that exhibited significant anxiolytic properties. TLC evidenced that flavonoids comprise the major group of compounds present in the active fraction. By comparing the R_f value of standard Quercetin with that of ethylacetate fraction, it was confirmed that quercetin was found to be the major compound present in ethylacetate fraction of methanol extract.

Various *in vivo* studies have recognized flavonoids as novel type of ligand with anti-anxiety outcomes. Behavioral tests in rodents have explored anxiolytic effects of different flavones (e.g., chrysin and apigenin) obtained from medicinal plants. The biological effect produced by these compounds is due to the modulation of GABA (γ-amino butyric acid) ergic system.[20] Neuroprotective manifestation of flavonoids has been attributed to their general bioavailability and *in vivo* occurrence in the brain.[21] Quercetin, one of the flavonol found in large number of herbals, act as a monoamineoxidase A and B (MAO A and B) inhibitor.[22] Research has revealed that substances acting as MAO modulators elicit behavioral alterations in rodents by modifying monoamine level in brain, and consequently display an anxiolytic effect.[20] Recent findings have proposed that MAO-inhibition and enhancement in GABA ergic activity may be the fundamental mechanism responsible for anxiolytic activity of quercetin liposomes administered via nasal route.[23]

Result of the present study indicated that the methanolic leaf extract of *A. conyzoides* had central anxiolytic effects. The phytoconstituent that may be responsible for the observed anti-anxiety effect has been detected in ethylacetate fraction of methanol extract and identified as a well-known compound, Quercetin, a flavonol.

CONCLUSION

In conclusion, the present study validates the traditional claim of *Ageratum conyzoides* as anxiolytic drug as methanol extract of leaf produced a significant anti-anxiety effect. Chemical fractionation identified quercetin as one of the major compound that may be responsible for the observed anti-anxiety effect of plant. Since there is a necessity for new safer and cost effective anxiolytic compounds having few side effects as compared to synthetic medication, *A. conyzoides* can act as a potential lead for drug development.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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Highlights of Paper

- *Ageratum conyzoides* Linn., commonly known as Jangli Pudina,is an important ethnomedicinal plant belonging to sunflower family i.e. Asteraceae.
- The leaves of the plant were found to be anxiolytic at 200 mg/kg p.o. dose.
- Anti-anxiety effect of the extract may be due to the presence of flavonoids in the fraction (obtained from extract) as confirmed by TLC.

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- **Mrs.Sarabjit Kaur:** Presently working as Assistant Professor in the Department of Pharmaceutical Sciences,Guru Nanak Dev University, Amritsar, Punjab, India. She has six publications to her credit of National and International repute.She has also presented six papers in national and international conferences.

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Assessment of Cytotoxicity of Smokeless Tobacco (Shammah) In Hepg2 and WRL68 Cells Line

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ABSTRACT

Shammah is a traditional form of chewing tobacco [Smokeless tobacco, (ST)] that is commonly used in the Middle east specially Saudi Arabia (KSA), Yemen and Sudan. The cytotoxicity of Sudanese and Yemenis ST hexane and methanol extracts was evaluated using MTT assay. Annexin-V assay has been used to detect the induction of apoptosis. Luminescence based assay also been conducted to check the level of caspases enzyme. The involvement of cell cycle check point arrest has been performed using flow cytometry analysis. The current study found that ST has the capacity to induce cell toxicity in human liver cells. The inhibitory capacity of ST in HepG2 and WRL 68 has been found to be 151 ± 2.5 and 305 ± 11.5 μg/ml for 24 h. An early apoptosis induction in HepG2 cells was observed by annexin V assay, which clearly exhibited significantly increased early and late apoptosis phases both at 24 and 48 h. Both the caspases-8 and-9 level was found to be increased by the introduction of ST to HepG2 cells significantly ($p < 0.05$). Moreover the ST extract was able to arrest the cell cycle check point at G2/M phase. A significantly increasing pattern of hypodiploid phases of cells also been observed, which confirm the apoptosis induction again. Collectively, results presented in this study demonstrated that the ST, which is used as a euphoritic substance of abuse also, has significant level of toxicity in human cells. Moreover the mode of cell death was found to be though programmed cell death which is closely associated with cell cycle arrest.

Key words: Hepatotoxicity, *In vitro* models, Saudi Arabia, Smokeless tobacco, Substance Absue Research Centre, Shammah.

INTRODUCTION

Smokeless tobacco is an unburned tobacco products that chewed in the oral cavity.[1,2] The unwanted harmful effects associated with the chewing of ST include cancerous and leukoplakian lesions of the oral cavity, periodontal disorders, and nicotine addiction. Immune-dysregulation of immune cells and their components may play a significant role in the progression of some unfavorable health effects associated with ST use. Some evidences also suggesting reproductive outcomes also exist in conjunction with ST use. Cardiovascular endothelia and blood pressure may also be impacted by ST use. Nitrosamines as tumorigenic products were also detected in ST products within allowed levels of concentrations.[3-8] These carcinogens have been extracted and chemically identified from ST samples. On the other hand, mutagenesis of ST have well been established using *in vivo* and *in vitro* models,[9,10] Cytotoxicity of ST on oral cell lines was observed *in vitro* to include inhibition of cellular proliferation, apoptotic cell death and involvement of inflammation pathways.[11]

Aqueous extract of ST at a single dose was previously found to cause oxidative stress in rat's liver causing increased levels of malondialdehyde. An earlier research showed treatment of HOK-16B cells with ST extract caused cell death. These findings provide an established scientific explanation to the ulceration and inflammation observed in people using ST at a new site within 2 days.[12-17]

Shammah is a traditional form of chewing tobacco [Smokeless tobacco, (ST)] that is commonly used in the Middle East specially Saudi Arabia (KSA), Yemen

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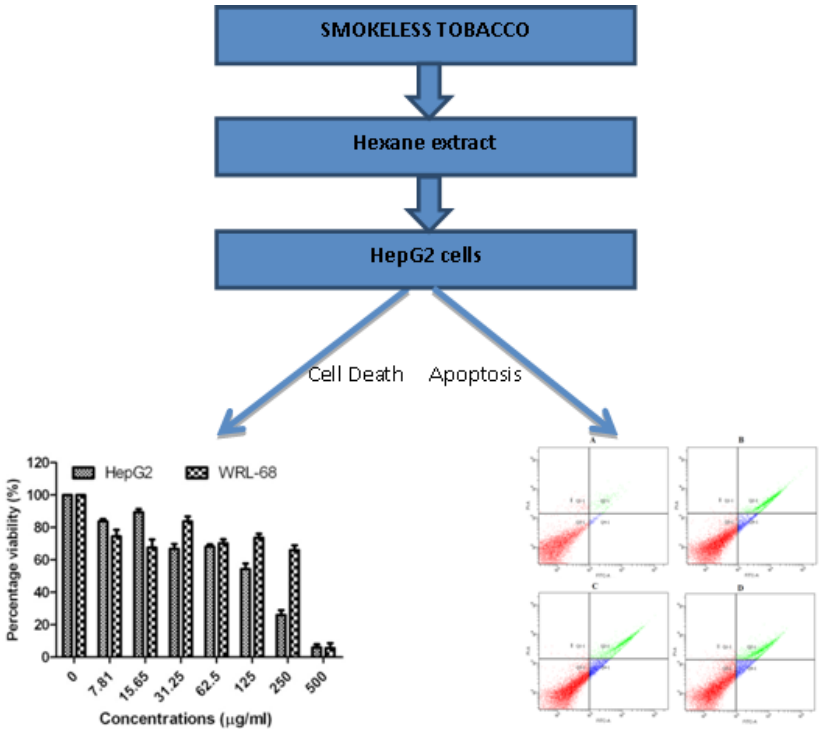

Graphical Abstract

and Sudan. In Sudan, about 40% of males and 10% of females use toombak (local name of ST).[18,19] Today, the trade of certain types of ST, such as moist snuff, is also illegal in several countries, e.g., in Saudi Arabia, Japan, Hong Kong, Singapore, Australia and New Zealand.[20] The There is various botanical taxonomy for tobacco plants. Therefore, the toxicity of these tobacco species and their health impacts may be reasonably different. To the best of our knowledge no studies were conducted to explore the hepatotoxicity of polar and non-polar extracts of ST using well-established *in vitro* models of liver toxicity. Therefore, the cytotoxicity of Sudanese and Yemenis ST hexane and methanol extracts was evaluated using established *in vitro* models of liver toxicity.

MATERIAL AND METHODS

Smokeless Tobacco Samples

Samples from Sudan and Yemen were obtained from exclusive shops in Khartoum (Sudan) and Jazan (Saudi Arabia), respectively.

Extraction procedure

Both Sudanese and Yemenis ST powder were added with enough quantity of hexane and macerated for three days with occasional shaking. The extracts were then filtered through filter paper Whatman® No. 41 and the residue were exposed to methanol for the polar extracts. The extraction procedure was same as done with hexane to obtain non-polar extracts. The filtrate was evaporated to dryness using (Buchi, R-210, Postfach, Switzerland) and store in refrigerator (-20ºC) until used.

Determination of cytotoxicity

Both HepG2 and WRL cells lines were purchased from ATCC, USA. Cells were plated and incubated until it reaches attachment and confluency. The medium was aspirated off and replaced with fresh medium (200 µl) containing ST extract of different concentrations in 96-well microplate except the last row which was left as a control. The plates were incubated at 37°C, 5% CO_2, for 24, 48 and 72 h. MTT containing medium was removed and replaced with 200 µl DMSO per well. The plate was mixed until the formazan crystals were dissolved and the plates were read on microtiter plate reader at 570 nm. The IC_{50} is generated from the dose-response curves for each cell lines. Three replicates in 96-well plates were analyzed for each cell type, concentration, and exposure period.

Cell cycle analysis by flow cytometry

The cell cycle phase arrest was studied using flow cytometry. HepG2 cells were plated and treated with ST. After 24 h, the cells were harvested in centrifugal tubes

and centrifuged at 3000 rpm for 5 min and fixed with 90% ethanol after washing with phosphate buffered saline (PBS) and refrigerated at -30°C. On the following day, the fixed cells were centrifuged at 3000 rpm for 5 min and the system was resuspended with 1 ml PBS. This washing process was repeated twice. One hundred microliter of 200 µg/ml DNAse free RNAseA enzyme was added to the cells and incorporated at 37°C for 30 min. The cells then were stained with 100 µl of 1mg/ml of propidium iodide and left at room temperature for 10 min. The cells were immediately analyzed using the FACS Canto II Becton-Dickinson Flowcytometryby analyzing at least 10,000 cells per sample. The percentage of cells in G1, S and G2 phases were analyzed by Mod Fit LT software (Verity Software House, Topsham, ME).

Caspase-8 and 9 activity assay

Caspase-8 and -9 activities were measured using luminescence-based assay, Caspase-Glo™ 8 Assay, and Caspase-Glo™ 9 Assay (Promega, US). Cells were cultured in 96-well culture plates in 50 µl of RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS) and incubated for 24 h. Cells were then treated with different concentrations of ST extracts. At the end of incubation, 100 µl of assay reagent was added to be incubated for 1 h at room temperature. Luminescence was measured using a microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

Annexin V assay

Cells (1×10^5 cells/ml) were exposed to ST for 24, 48 and 72 h and an Annexin V assay was done according to BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, Clon Tech, California, USA) instructions. In short, cells were centrifuged at 200 x g for 10 min. Supernatant was removed and cells were rinsed with 1 x binding buffer and re-suspended in 200 µl of binding buffer from the kit. Cells were stained with 5 µl of Annexin V and 10 µl of propidium iodide (Sigma, Saint Louis, Missouri, USA) and incubated in the dark for 15 min at room temperature. Using a FACS Canto II Becton-Dickinson flow cytometer, analysis was performed with at least 10,000 cells/sample. The reaction volume was brought to 500 µl for the flow cytometry analysis with the aid of binding buffer available from the kit. Cells treated with DMSO (0.1%, v/v) served as control.

RESULTS

Cytotoxicity on Liver Cells

The effect of ST samples on cell growth inhibition was expressed as an IC_{50} value. Only hexane extract of Yemenis sample showed toxicity on liver cells. Cellular proliferation following 24 h of exposure to ST samples showed considerable inhibition in ST-treated cells compared to non-treated cells (controls). The proliferation of ST-treated cells decreased as the ST concentration with IC_{50} of 151 ± 2.5 and 305 ± 11.5 µg/mL on HEPG2 and WRL-68, respectively for the hexane extract of Yemen shamma (Figure 1).

Annexin V

To determine the occurrence of apoptosis, annexin-v assay has been used. The HepG2 cells have been treated with the IC_{50} concentration of ST for 24, 48 and 72 h. The results had

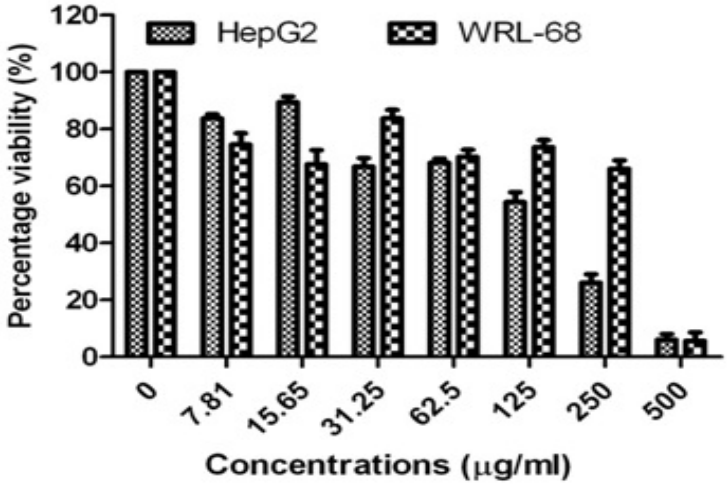

Figure 1: ST sample hexane extract was tested in HepG2 and WRL-68 cell lines. Cell viability was determined by MTT assay. The test agent induced cell cytotoxicity in a time-dependent manner. These dose titration curves allowed the IC_{50} of the test agent towards different cell lines to be determined. The proliferation of ST-treated cells decreased as the ST concentration with IC_{50} of 151 ± 2.5 and 305 ± 11.5 µg/mL on HEPG2 and WRL-68, respectively

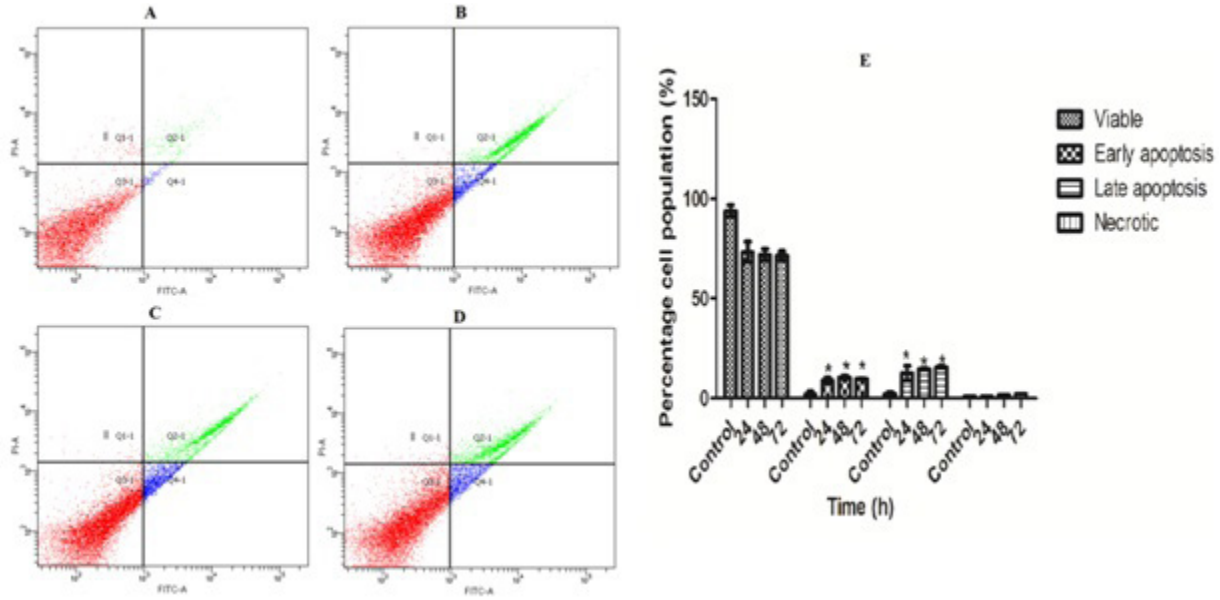

Figure 2: Annexin-V-FITC staining HepG2 cells were treated IC_{50} concentration of ST for 24, 48 and 72 h. After staining with FITC-conjugated Annexin V and PI, cells were analyzed by flow cytometry. Control cells received no drug treatments (A). (B–D) The effects of 24, 48 and 72 hr exposure (respectively) of HepG2 cells. The results showed statistically significant ($p<0.05$) (E)

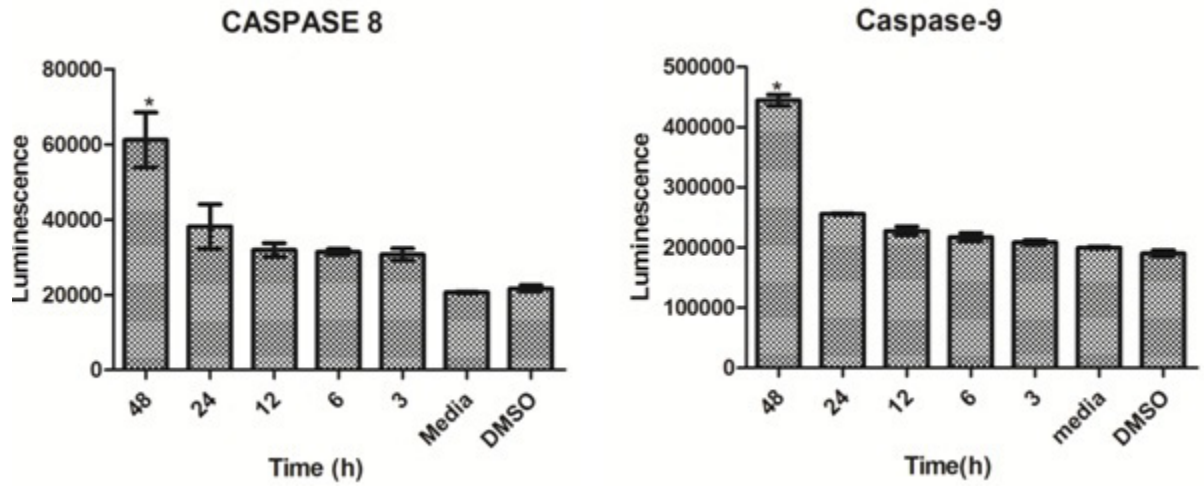

Figure 3: The luminescence-based assay of caspase-8 and caspase-9 in HepG2 cells treated and untreated with ST hexane extract at different time points. Cells were cultured in RPMI 1640 media maintained at 37°C and 5% CO_2

'*' Indicates a significant difference from the control ($p<0.05$)

showed that the ST extract inhibited the proliferation of HepG2 cells significantly compared to control. As shown in (Figure 2), the apoptosis induction in the cells began after being treated for 24 h. For the untreated control, 89% ± 4.5 cells were viable and only 3 ± 0.22% cells were in the early apoptosis stage. But in 24 h the treated cells early viable cells phase reduced significantly to 73± 5.6% with a rise in early apoptosis cells to 10 ± 1.1% and 15 ± 2.0% late apoptosis cells. Even though the apoptosis phase was significant, there was no time depended increase in the apoptosis cells at 48 and 72 h compared to 24 h treatment. These results indicated that the ST allowed the translocation of PS to occur and hence induced early apoptotic induction in HepG2 cells.

Induction of caspases activation

Both the caspases under investigation were found to be induced while the treatment, and were found to be time-dependent. High levels of both caspase 8 and 9 were

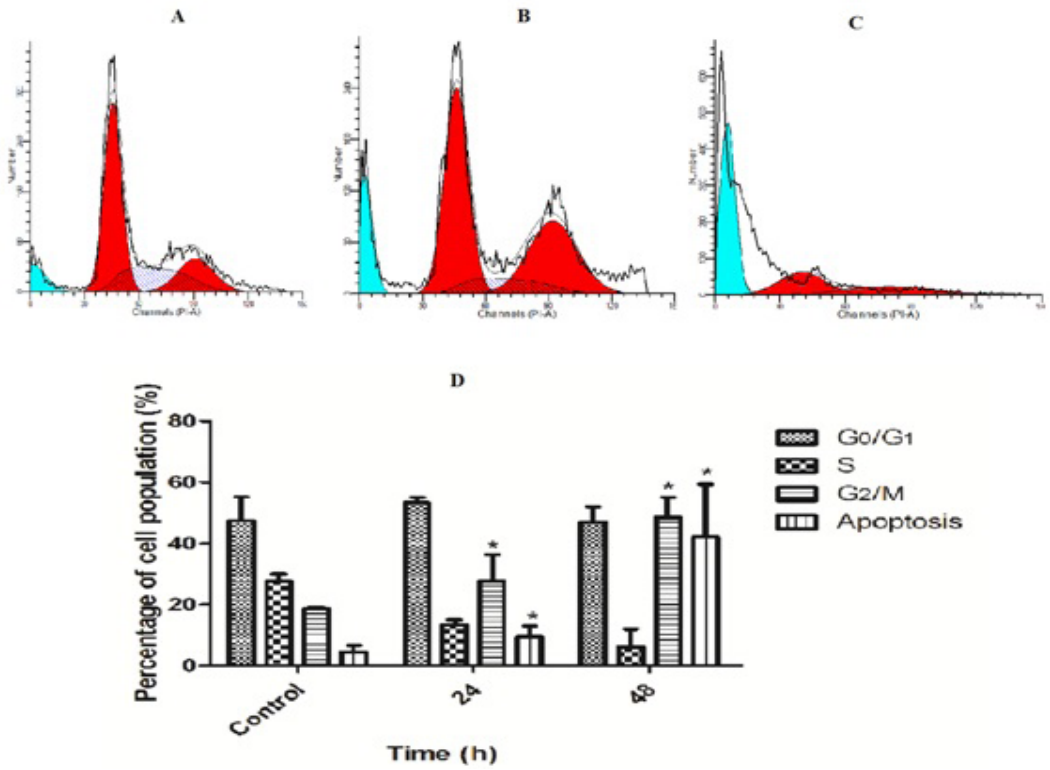

Figure 4: DNA analysis of ST hexane extract-treated HepG2 cells. Cells were exposed to IC50 concentrations at 24 h (B) and 48 (C) and control (A) were tested for DNA content of Sub-G0/G1, G0/G1, S and G2/M phase. Induction of G2/M arrest in the cell cycle progression of cells was observed (D).

'*' Indicates a significant difference (p< 0.05)

found at the highest time period of 48 h with a significance difference from control ($p<0.05$). But the activation of caspases was not significantly induced at early time periods. These results confirmed the activation of caspases was involved in the cell death induced by ST (Figure 3).

ST inhibits the HepG2 cell proliferation and arrest G2/M phase cell cycle

To check the involvement of cell cycle progression/arrest and apoptosis the cell cycle analysis has been performed. The results from the current study established that ST arrested the cell cycle at G2/M phase of the cell phase and induced significant apoptosis ($p<0.05$). The results shown in (Figure 4) indicated that the cell cycle arrest happened in time dependently, with a rise in G2/M phase to 30 ± 4.61and 48 ± 2.9% for 24 and 48 h respectively. Moreover the hypodiploid (sub G1/apoptosis), which is considered as the apoptosis phase has also rose up to 42 ± 6.7%.

DISCUSSION

The main toxic effects observed with the substance of abuse are carcinogenicity.[21] It is difficult to predict the particular agent causing this effect due to the fact that many a times their will be a mixture of substance, especially used by traditional practice. Shammah is one of the unburned tobacco products that chewed in the oral cavity. Even though officially banned in Saudi Arabia, it is chewing due to its psychoactive properties.[22] It has been found that chewing of Shammah has significant effect in mutagenicity and carcinogenicity. Previous studies had showed that the Shammah extract has the capacity to produce ROS in oral keratinocyte cells, capacity to increases in fragmentation of genomic DNA, and apoptotic mode of cell death.[23] But there is no report on the effect of Shammah in liver cells.

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death.[24,25] Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. Positive functions of apoptotic genes have been well established in a large number of biological contexts, including their role in eliminating damaged and

potentially cancerous cells. Evidence has suggested that proapoptotic proteins like caspases can induce proliferation of neighboring surviving cells to replace dying cells. This process is known as apoptosis-induced proliferation.[26] Whether the STE administration has the same kind of effect is unclear. In this study, the liver cells have been used to check it ability to undergo apoptosis. Out of HEPG2 and WRL-68 cells, HepG2 cells have been found to be more susceptible to toxicity produced by STE. It shows that the cytotoxicity produced by STE is cell specific and may be followed by specific cell signaling.

The Annexin V assay was used in the research as an initial attempt to investigate the probable mechanism of cell death responsible for the cytotoxic effect of the STE towards HepG2 cells. Phosphatidylserine (PS) externalization during apoptosis, a universal phenomenon during apoptosis is not limited in mammalian cells, but also occurring in both insect and plant cells.[27] Annexin V was found to bind specifically to PS, located at the outer membrane leaflet of cells in the presence of calcium. The present study demonstrated that treatment with STE is able to induce cell death via apoptosis. Even though the 24 h results had showed a significant amount of PS exposure there was no time dependent increase in the treatment. This may be due to cytostatic effect produced by the cells itself against the toxicity of STE.[28]

It is very apparent that cell cycle arrest happened concurrently with cell cycle arrest[29,30] In this study we have used flow cytometric measurements to assess apoptotic cell death using propidium iodide as the probe. At the time of cellular death, nuclei of the cells are stained with the fluorescent dye propidium iodide. The results clearly show that STE can induce time-dependent cell cycle arrest together with apoptosis in HepG2 cells. More over the increased hypo diploid cells observed in the cell cycle analysis further confirm the apoptosis induction.[31]

Caspases plays a vital role in initiating and executing apoptosis process.[32] Generally apoptosis happens though both intrinsic and extrinsic pathways. Both pathways have different caspases initiator. Caspase 8 and 9 plays the role of initiation in intrinsic and extrinsic pathways respectively.[33] At the downstream pathways Caspases are executioners of apoptosis that can dismantle a cell within hours. Hence the current study studied the role of caspases in STE in HepG2 cells. Both the caspases were found to be activated especially during the late time period of STE treatment.

In summary, we have established that the smokeless tobacco has the capacity to induce apoptosis and cell cycle arrest in the liver cells. The cell death was closely associated with caspases activation and typical apoptosis form of death. This suggests that Shammah has significant levels of toxicity with definite cell death mechanism. It needs further in-depth mechanism studies and animal based experiments.

CONCLUSION

In summary, we have established that the smokeless tobacco has the capacity to induce apoptosis and cell cycle arrest in the liver cells. The cell death was closely associated with caspases activation and typical apoptosis form of death. This suggests that Shammah has significant levels of toxicity with definite cell death mechanism. It needs further in-depth mechanism studies and animal based experiments.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this work.

ACKNOWLEDGEMENTS

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Highlights of Paper

- Shammah is a traditional form of chewing tobacco.
- Hexane extract of Shammah induced cell death and apoptosis in HepG2 cells.
- The cell death happened together with Caspases induction and cell cycle arrest.
- Cell cycle arrest has been happed at G2.M phase.

Author Profile

- **Dr.Siddig Ibrahim Abdelwahab** is Associate professor in Jazan University, Jazan, KSA. He graduated from Faculty of Veterinary Sciences- University of Khartoum in the field of Veterinary Medicine in the year 1999 and obtained his Master of Science and Doctor of Philosophy Degrees in Pharmacology from University Putra Malaysia, Malaysia in the years 2001 and 2008 respectively. He also had opportunity to join as Head of the Biomedical Research Unit, (Medical Research Centre, and Substance Abuse Research Centre) and Core Laboratory, Medical Research Centre,Jazan, Saudi Arabia. In his attempt to share his expertise, he acquired an overall H-index of 19 with his high number of research publications (130) and two patents filed. He is active member of four scientific communities and is been considered as potential reviewer for more than 10 biomedical journals. His excellence in research also extends to statistical analysis.

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GC-MS Analysis of Phytocomponents in, Pet Ether Fraction of *Wrightia tinctoria* Seed

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ABSTRACT

Introduction: *Wrightia tinctoria* R.Br. (Family: Apocynaceae) commonly called "Indrajau" is well known in Indian traditional system for its traditional uses. **Materials and Methods:** The present investigation was carried out to determine the possible bioactive components of plant seed ethanolic extract, pet ether fraction using GC-MS analysis. 22 components were identified from pet ether fraction obtained from elution of ethanolic extract packed in silica column. **Results:** The prevailing compounds from fraction F6to F9 were [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester (21.39%) , Trilinolein (7.74%), 2-Myristynoyl pantetheine (18.07%), 9-Octadecen-12-ynoic acid, methyl ester (4.46%), 1Hexadecanol,2-methyl (3.77%), Cyclopropane tetradecanoic acid, 2-octyl-, methyl ester (2.36%), 1b, 4a-Epoxy-2H-cyclopenta [3,4] cyclopropa [8,9]cycloundec [1,2-b]oxiren-5 (6H)-one, 7-(acetyloxy) decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl (38.91%), Geranyl isovalerate (23.58%), cis-13-Octadecenoic acid (5.91%), Quassin (3.82%), cis-10-Heptadecenoic acid (3.08%), 9,12,15-Octadecatrienoic acid 2-phenyl-1, 3-dioxan-5-yl ester (31.50%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-2,3-dihydroxypropyl ester (14.35%), Cyclopropanebutanoic acid, 2-[[2-[[2- [(2-pentylcyclopropyl) methyl] cyclopropyl] methyl] cyclopropyl] methyl]-, methyl ester (10.13%), 6,9,12,15-Docosatetraenoic acid, methyl ester (3.39%), 9,12-Octadecadienoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, trans-(2.73%), 9,12-Octadecadienoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, cis-(4.34%), Ursodeoxycholic acid (7.14%), Bufa-20,22-dienolide, 3-(acetyloxy)-14,15-epoxy-16-hydroxy-, (3á,5á,15á,16á)-(4.75%), 5H-Cyclopropa [3,4] benz [1,2-e]azulen-5-one, 9a (acetyloxy)-1,1a,1b,4,4a,7a,7b,8,9,9a-de cahydro-4a,7b,9-trihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,[1aR-(1aà,1bá,4aá,7aà,7bà,8à,9á,9 aà)]-(6.59%), Docosahexaenoic acid, 1,2,3-propanetriyl ester (10.86%), Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à)-(4.40%) found as the major components. **Conclusion:** It could be concluded that, *Wrightia tinctoria* contains various bioactive compounds. So it is recommended as a plant of phytopharmaceutical importance.

Key words: Bioactive components, Ethanolic extract, GC-MS, Indrajau, *Wrightia tinctoria*.

INTRODUCTION

Plants are used medicinally in different countries, and they are the source of many potent and powerful drugs. Plants have been an important source of medicine with qualities for thousands of years. Mainly on traditional remedies such as herbs for their history, they have been used as popular folk medicines. It has been shown that *in vitro* screening methods could provide the needed preliminary observations necessary to elect crude plant extracts with potentially useful properties for further chemical and pharmacological investigations.[1] GC–MS is the best technique to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids, ester, alkaloids, steroids, amino and nitro compound etc.[2] *Wrightia tinctoria* R.Br. (Family: Apocynaceae) commonly called "Indrajau" is distributed throughout the world and occurs abundantly in India. It is a deciduous tree with white fragrant flowers. The seeds and bark of this plant are used in Indian traditional medicine as anti-diarrheal and anti-dysenteric.[3]

Medicinal uses: Ethno medically, the bark of this plant is used as a galactagogue, to treat abdominal pain, skin diseases and wounds, as an antipyretic, antidysenteric, antidiarrheal and antihemorrhagic agents and as an antidote for snake poison. Seeds of this plant are also used as an aphrodisiac.

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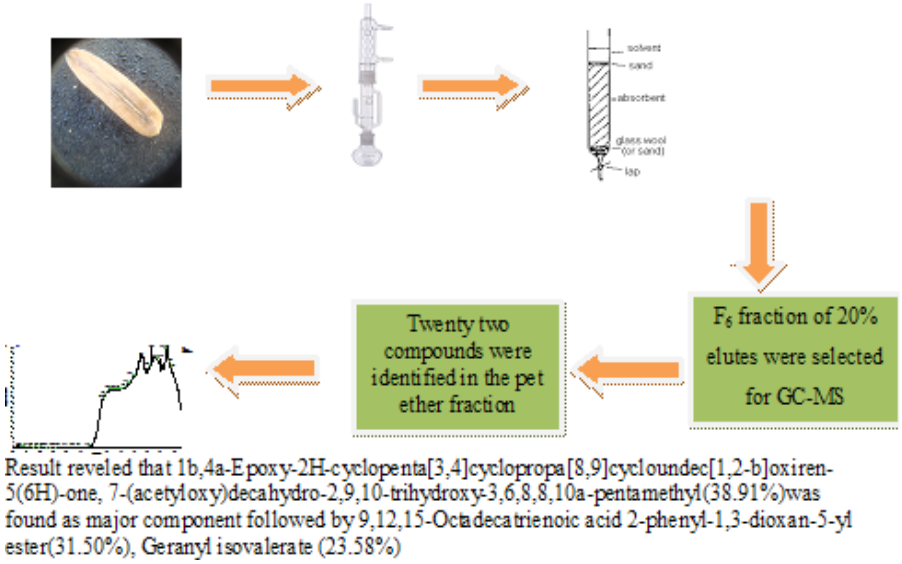

Graphical Abstract

In view of the reported severe health hazards of estrogen, such as increased risk of endometrial hyperplasia and carcinoma breast cancer and thromboembolic diseases. A large number of natural products showing promising antifertility activity in preliminary studies could not be pursued due to their associated estrogen-agonistic activity.[4]

MATERIALS AND METHODS

Collection of plant material

The *Wrightia tinctoria* seed were collected from its natural habitat in Jharkhand and identified by Botanist at (NBRI) national botanical research institute, Lucknow, Voucher specimens were preserved at the Herbarium of the institute.

Preparation of extract and fraction

The seeds of *Wrightia tinctoria* washed dried and powered, extracted with per ether, chloroform, ethanol and water in successive session in soxhlet percolator. Extractable value calculated by evaporating the solvent in vacuum evaporator. In pet ether no crystalline component obtained instead get rubbery material, while chloroform and ethanolic extracts are semisolid in nature. Ethanolic extract packed in silica column and column is eluted with 10%, 20%, 30%, 40%, 50% pet ether and chloroform mixture solvents. The F_6 fraction of 20% elutes were selected for GC-MS analysis.

GC–MS analysis

MS sample has been analyzed on THERMO Scientific TSQ 8000_(triple quadrupole Gas Chromatograph-Mass Spectrometer) This mass spectrometer comes paired with the TRACE 1300 GC along with Auto-sampler for automated sample. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min. Ion Source Type: EI source programmable to 350°C. Scans: 731. Condition is: Initial temperature is kept at 60°C, then temperature is increased to 280°C with a max ramp of 90°C/min. Then temperature is allowed to cool down. MASS range was kept between 50-500 m/z. injection volume 10 (µl). Run Time: 2.48 min, This GC-MS is equipped with NIST Library.

Identification of Components

Interpretation on mass spectrum of GC–MS was done using the database of National Institute of standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and molecular formula and probability of the components of the test materials were ascertained.

RESULTS AND DISCUSSION

GC–MS analysis

The components present in the ethanolic extract of seed pet ether fraction was identified by GC–MS (Figures 1-6). The prevailing compounds from fraction F_6 to F_9 were [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester (21.39%) , Trilinolein (7.74%), 2-Myristynoyl pantetheine (18.07%), 9-Octadecen-12-ynoic acid, methyl ester (4.46%), 1Hexadecanol, 2-methyl

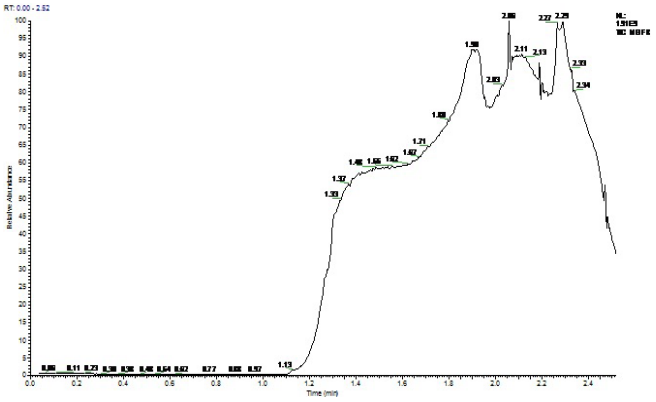

Figure 1: GC-MS total ion current chromatogram of pet ether faction

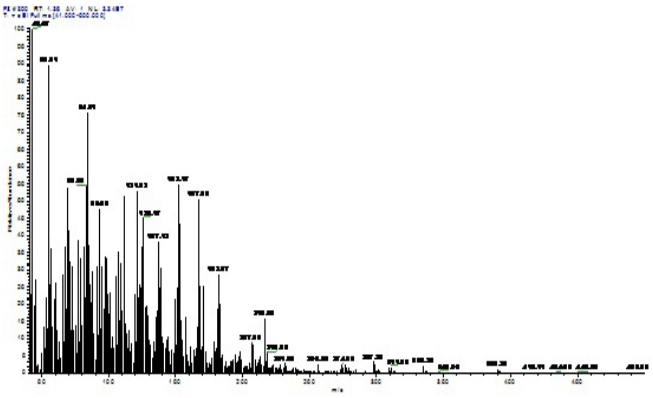

Figure 2: GC-MS Spectra 1

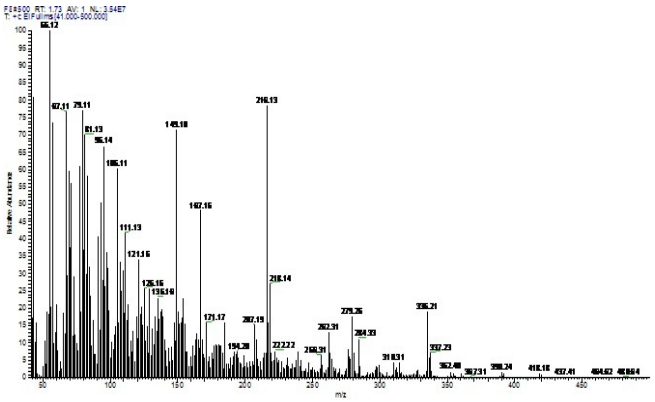

Figure 3: GC-MS Spectra 2

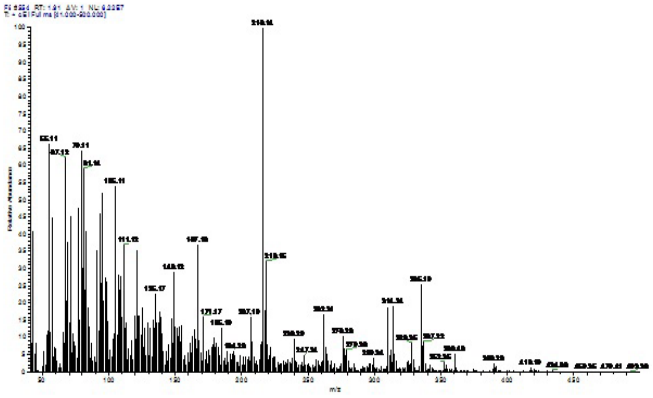

Figure 4: GC-MS Spectra 3

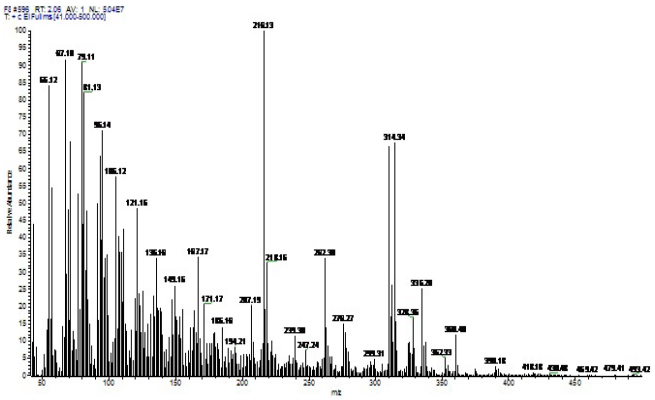

Figure 5: GC-MS Spectra 4

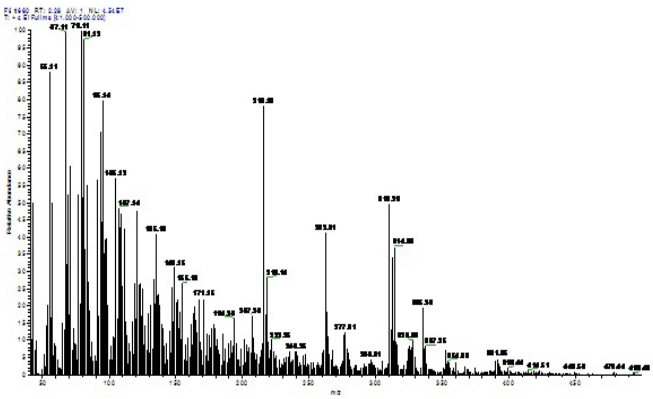

Figure 6: GC-MS Spectra 5

(3.77%), Cyclopropane tetradecanoic acid, 2-octyl-,methyl ester (2.36%), 1b, 4a-Epoxy-2H-cyclopenta[3,4] cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl (38.91%), Geranyl isovalerate (23.58%), cis-13-Octadecenoic acid (5.91%), Quassin (3.82%), cis-10-Heptadecenoic acid (3.08%), 9,12,15-Octadecatrienoic acid 2-phenyl-1,3-dioxan-5-yl ester (31.50%), 9, 12, 15-Octadecatrienoic acid, (Z,Z,Z)-2,3-dihydroxypropyl ester (14.35%), Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl] methyl]cyclopropyl]methyl]-,methyl ester (10.13%), 6,9,12,15-Docosatetraenoic acid, methyl ester (3.39%), 9, 12-Octadecadienoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, trans-(2.73%), 9,12-Octadecadienoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, cis-(4.34%), Ursodeoxycholic acid (7.14%), Bufa-20,22-dienolide, 3-(acetyloxy)-14,15-epoxy-16-hydroxy-,

Table 1: Components detected in pet ether fraction

Compound name (con%)	Pharmacological activity
Trilinolein (7.74%)	Anti-ischemic, Antiarrhythmic, and Antioxidant
2-Myristynoyl pantetheine(18.07%)	---
9-Octadecen-12-ynoic acid, methyl ester (4.46%)	Immunotoxicity effects, and Antioxidant activity
1-Hexadecanol, 2-methyl (3.77%)	Antimicrobial
Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester (2.36%)	Antimicrobial
[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester (21.39%)	Antibacterial
1b,4a-Epoxy-2H-cyclopenta[3,4] cyclopropa[8,9] cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl(38.91%)	---
Geranyl isovalerate (23.58%)	Antimicrobial
cis-13-Octadecenoic acid (5.91%)	dopaminergic stimulatory activity
Quassin(3.82%)	Antiplamodial and antiviralactivity
cis-10-Heptadecenoic acid (3.08%)	Anticancer
9,12,15-Octadecatrienoic acid 2-phenyl-1,3-dioxan-5-yl ester(31.50%)	---
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-2,3-dihydroxypropyl ester (14.35%)	Analgesic, Antipyretic, Anticonvulsant, Antiseptic
Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]	
cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester(10.13%)	---
6, 9, 12, 15-Docosatetraenoic acid, methyl ester (3.39%)	Anticholesterol compound
9, 12-Octadecadienoic acid, (2-phenyl-1, 3-dioxolan-4-yl) methyl ester, trans-(2.73%)	---
9, 12-Octadecadienoic acid, (2-phenyl-1, 3-dioxolan-4-yl) methyl ester, cis-(4.34%)	---
Ursodeoxycholic acid (7.14%)	---
Bufa-20, 22-dienolide, 3-(acetyloxy)-14, 15-epoxy-16-hydroxy-, (3á,5á,15á,16á)-(4.75%)	---
5H-Cyclopropa[3,4] benz [1,2-e]azulen-5-one, 9a-(acetyloxy)-1,1a,1b,4,4a,7a,7b,8,9,9a-de cahydro-4a,7b,9-trihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,[1aR-(1aà,1bá,4aá,7aà,7bà,8à,9á,9aà)]-(6.59%)	---
Docosahexaenoic acid, 1, 2, 3-propanetriyl ester(10.86%)	---
Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à)-(4.40%)	---

(3á,5á,15á,16á)-(4.75%), 5H-Cyclopropa [3,4] benz [1,2-e] azulen-5-one, 9a (acetyloxy)-1,1a,1b,4,4a,7a,7b,8,9,9a-de cahydro-4a,7b,9-trihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,[1aR-(1aà,1bá,4aá,7aà,7bà,8à,9á,9aà)]-(6.59%), Docosahexaenoic acid, 1,2,3-propanetriyl ester(10.86%), Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à)-(4.40%) found as the major components.

Twenty two compounds were identified in the pet ether fraction; principal components with their % concentration and pharmacological activity were presented in (Table 1).

Result reveled that 1b, 4a-Epoxy-2H-cyclopenta [3,4] cyclopropa [8,9] cycloundec [1,2-b]oxiren-5 (6H)-one, 7-(acetyloxy) decahydro-2, 9, 10-trihydroxy-3,6,8,8,10a-pentamethyl (38.91%) was found as major component followed by 9,12,15-Octadecatrienoic acid 2-phenyl-1,3-dioxan-5-yl ester (31.50%), Geranyl isovalerate (23.58%) .

CONCLUSION

In the present study, 22 components were identified from seed alcoholic extract, pet ether fraction by

Gas Chromatography–Mass Spectrometry (GC–MS) analysis. The presence of various bioactive compounds justifies the use of this plant for various ailments by traditional practitioners. However, isolation of individual constituents and subjecting it to biological activity will definitely give fruitful results. It could be concluded that, *Wrightia tinctoria* contains various bioactive compounds. So it is recommended as a plant of phytopharmaceutical importance. However, further studies are needed to undertake its bioactivity and toxicity profile.

CONFLICTS OF INTEREST

Authors declared no conflict of interest.

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ABBREVIATION

GC–MS: Gas Chromatography–Mass Spectrometry.

NIST: National Institute of standard and Technology.

Highlights of Paper

- *Wrightia tinctoria* R.Br. (Family: Apocynaceae) commonly called “Indrajau” is well known in Indian traditional system for its traditional uses.
- The components present in the ethanol extracts of *Wrightia tinctoria* seed pet ether fraction was identified by GC–MS.
- Twenty two compounds were identified in the pet ether fraction.
- Result reveled that 1b,4a-Epoxy-2H-cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl(38.91%)was found as major component followed by 9,12,15-Octadecatrienoic acid 2-phenyl-1,3-dioxan-5-yl ester(31.50%), Geranyl isovalerate (23.58%).
- *Wrightia tinctoria* contains various bioactive compounds. So it is recommended as a plant of phytopharmaceutical importance

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